PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION CONCERNING
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To:

United States Patent and Trademark
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Date of mailing:	
12 December 1994 (12.12.94)	
International application No.: PCT/NO93/00136	International filing date: 10 September 1993 (10.09.93)
Applicant: FODSTAD, Øystein et al	

The International Bureau transmits herewith the following documents and number thereof:

_____ copy of the international preliminary examination report and annexes (Article 36(3)(a))

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorised officer:

I. Britel

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PATENT COOPERATION TREATY

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
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Date of mailing: 11 May 1994 (11.05.94)	
International application No.: PCT/NO93/00136	Applicant's or agent's file reference: Fod 1 P93496 HV
International filing date: 10 September 1993 (10.09.93)	Priority date: 14 September 1992 (14.09.92)
Applicant: FODSTAD, Øystein et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

14 April 1994 (14.04.94)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
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Authorized officer:

C. Combaz

Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

To:

ONSAGERS PATENTKONTOR AS
P.O. Box 265 Sentrum
N-0103 Oslo
NORVEGE

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing: 11 May 1994 (11.05.94)	
Applicant's or agent's file reference: Fod 1 P93496 HV	IMPORTANT INFORMATION

International application No.	International filing date	Priority date
PCT/NO93/00136	10 September 1993 (10.09.93)	14 September 1992 (14.09.92)

Applicant: FODSTAD, Øystein

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Office of its election:

AT,AU,BB,BG,BR,BY,CA,CZ,DE,DK,EP*,FI,GB,HU,JP,KP,KR,KZ,LK,LU,MG,MN,MW,NL,NO, NZ,OA,PL,PT,RO,RU,SD,SE,SK,UA,US,VN

*AT,BE,DE,DK,FR,GB,IE,IT,LU,MC,NL,PT,SE

The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of the annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

Some offices have fixed time limits expiring later than the above-mentionned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent including, where applicable CH & LI, ES and GR, which cannot be elected since they are not bound by Chapter II.

With respect to Poland, a translation into Polish must always be furnished within 20 months from the priority date **where those 20 months expire before 1 March 1994**, even if Poland was elected for international preliminary examination before the expiration of 19 months from the priority date. Where those 20 months expire on or after 1 March 1994, the translation into Polish must be furnished, if Poland was elected for international preliminary examination before the expiration of 19 months from the priority date, before the expiration of 30 months from the priority date.

The International Bureau of WIPO
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1211 Geneva 20, Switzerland

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Authorized officer:

C. Combaz

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ONSAGERS PATENTKONTOR AS
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OLAV L. ROBSAHM, Dr.rer.nat. (A[redacted]m), fys.
GEIRR I. LEISTAD, Cand.mag. (Oslo), fys.
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Fnr: 934603729

[redacted]

European Patent Office
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Deres (Your) ref	Vår (Our) ref.	Dato
	Fod 1 P93496 KM/GT	26. august 1994

International application PCT/NO93/00136

The written opinion of 10 June 1994 is referred to.

The Examiner claims that the present application does not seem to satisfy the criterian set forth in Article 33 (3) PCT because the subject matter claimed does not involve an inventive step (Rule 65 (1) (2) PCT).

We are, however, of the opinion that the present application meets the requirements regarding inventive step, and are respectfully providing the following argumentation:

The invention described in Doc. D1 (WO-A-9204961) has a completely different object than the present invention, namely to separate substances from test media which consists of liquid (f.ex. blood). In the referred document molecular biological examinations are not mentioned, neither are analyses on biopsies from solid tissue, which is the aim of the present invention. It will therefore not be likely for an artisan in the field to anticipate the present method based on the technique learned from D1. The Examiner states that in D1 "a separate resuspension of the target cells is avoided and that analytical procedures as quantitative determinations may be performed on the magnetically immobilized colloid". The particles in the invention described in D1 are so small that it would be impossible to examine them visually as in the present invention. Therefore it is not possible, from the teaching of D1 to anticipate those kind of analyses which are possible to do with the cell particle complexes described in the present invention.

We will in the following elaborate on this.

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Magnetic particles:

The specification in D1 describes use of small (sub-micron) colloidal magnetic particles. On page 13, line 10 we refer "...sub-micron particle size, which is generally less than 200 nanometers (nm) (0,20 microns)". The present invention describes use of considerably larger particles (in the Examples it is used Dynabeads with a size of 4,5 microns) which is necessary to obtain visual detection and quantifysation in a microscope. Small colloidal particles could never have been used for this object. Contrary in document D1 it is claimed that for their object it is necessary with small particles to avoid that the particles precipitate out of the solution during the examination period. It is on page 7 and 9 argued for use of the small particles and against use of particles larger than 0,7 to 1.5 microns (page 4, first paragraph). It is difficult to see how an artisan in the field should think of using larger beads because, with their technique, larger beads would be of no use at all.

Magnetic field:

The method described in D1 uses so-called "magnetic field intensifying means" to obtain adequate magnetic power to isolate particles conjugated with target molecules or target cells. This is obtained by "magnetic wires or screens (i.e. the magnetic field intesifying means)" are "immersed substantially within the test medium" (page 9, line 32) and that "the receptor-magnetic particle conjugates and the magnetic field intensifying means are placed in a magnetic separator". This is a very complicated system which is not necessary for the separation technique described in the present invention. We regard it as very unfortunate for any test sample, among other thing when the isolated cells shall be grown sterile, that it should be necessary to immerse foreign subjects into the test media. In the present invention the magnetical system obtain adequate magnetical power in relation to the bead/cell conjugate with the location of a strong magnet outside the test tube without any special means, such as those described in D1. Thus it seems very unlikely that the teaching of D1 should lead the artisan in the field to anticipate the magnetic system described in the present invention since this was not possible with the size of the particle used in the invention described in D1.

Objects and methods of evaluation:

In the present application the object is to detect specific target cells visually in a simplest possible way in a microscope. Thereby it is possible to establish the presence of the target cells in addition to quantifying the target cells in relation to the total number of cells originally present in the medium. In the invention of document D1 the object is to perform a semi-quantitative determination of target molecules or cells, but this quantification is dependent upon using "enzyme-labelled immunoassays" (page 23,

line 20-30) with second[redacted] immunochemical reactions (e.g. enzym[redacted], fluorescent or chemiluminescent). To perform this quantification it will always be necessary to have a standard reaction as a control and a standard curve in which a known amount of the reactive target molecules are added. Such reactions are not necessary when using the present method. When the Examiner refer to page 23 in document D1 regarding quantification it may be due to that it is, in the present invention, stated that besides obtaining the main object (quantification) it is possible to use the isolated cells further for other examinations, for example immunological and especially molecular biological examinations. They thus represent possibilities given by the present method in addition to the visual microscopic quantification. Besides the specification in D1 it does not mention anything about molecular biological examinations. It seems therefore unlikely that such examinations should be anticipated from the teaching of D1.

In the specification of D1 several examples are describing the use of the method mentioned. In most of the occasions it is described isolation of immune/-hematological cells which thereafter have to be determined by a "sandwich-assay" with the reactions mentioned above. Hematological cells are excepted in the present application. In D1 it is for example mentioned detection of two other molecules, hCG and estradiol, both hormonal, cell produced molecules and not specific target cells in a mixed cell population. It should also be mentioned that the method D1 is suitable for examination (page 14, lines 5-6) of "appropriately prepared body fluids, such as blood, urine, sputum or secretions". In addition to tissue liquid the method of the present invention offers the possibility of identifying cells present in single cell suspensions prepared from solid tumors and several other types of pathological and normal tissue. It does not seem likely that methods for examination of solid tissue could be anticipated from a method which is restricted to liquid test samples.

Specificity:

Regarding quantification, the examples of the method taught in D1 shows very low specificity. Thus is it in example 1, table 1 (page 29) shown a separation effectivity of about 77% (minus background which is 11-13%). With the aim of the method described in the present invention a method with such low specificity could not have been used at all, since the applicants are dependent on that almost 100% of the cells with particle rosettes represent the target cells, especially when the object is to detect a small number of target cells mixed in a very high number of normal cells. The high unspecific binding in the method of D1 is further demonstrated in table 2, page 32. In an example on page 37, line 10, an example for detection of T-cells in peripherally blood is exhibited. In this example it is underlined that the mono-nuclear cells (PBMC) are

isolated before the test be performed. This is not necessary the method of the present invention. It seems to us completely unlikely that a method for quantification of a small number of cells should be anticipated from a known technique in which the specificity is so low as exhibited in the method of D1.

In the method described in D1 it is taught the use of Tamol 850 ("a suitable anionic polyelectrolyte") to reduce unspecific binding of colloid particles to the target cells. We have used a detergent for the same reason. The enormous difference in specificity between the method of D1 and the present method seems mainly to be due to other factors, but it can not be ruled out that the detergent is a better choise than Tamol.

We therefore respectfully ask the Examiner to re-evaluate the conclusion regarding the inventive step of the present invention in light of the above comments.

Regarding certain observations on the International Application we have performed the following amendments.

1. The subject matter of claim 1 is identified as "a method for" instead of "improved method for".

2. The document D1 is now identified in the description and the relevant background art disclosed therein is briefly discussed on page 3.

3. The PCT Application Nos. mentioned in the description are replaced by the corresponding publicly accessible publication numbers. This is performed on page 1 and 2.

4. Terms Tween, (page 11, claim 1.4.1) and Dynabeads (pages 19, 20 and 21) are identified as Trade Marks.

Yours faithfully,
Onsagers Patentkontor AS

Handled by: Kjell Myhre

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

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under Article 31 of the Patent Cooperation Treaty:

The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

CHAPTER II

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference
		Fod 1 P93496 HV
International application No. PCT/NO93/00136	International filing date *(day/month/year)* 10 September 1993 (10-09-93)	(Earliest) Priority date *(day/month/year)* 14 September 1992 (14-09-93)

Title of invention: Improved method for detection of specific target cells in specialized or mixed cell population and solutions containing mixed cell populations

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*	Telephone No.:
Fodstad, Øystein Frits Kiers v. 28 N-0383 OSLO NORWAY	Facsimile No.: Teleprinter No.:

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:
NO	NO

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Kvalheim, Gunnar
Åsstubben 13

N-0381 OSLO NORWAY

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:
NO	NO

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:

☐ Further applicants are indicated on a continuation sheet.

See Notes to the demand form

International application No.
PCT/NO93/00136

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

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[ ] Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the International Preliminary Examining Authority*

(i) [ ] to start the international preliminary examination on the basis of the international application as originally filed.

(ii) [X] to take into account the amendments under Article 34 of

- [ ] the description (amendments attached).
- [X] the claims (amendments attached).
- [ ] the drawings (amendments attached).

(iii) [ ] to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).

(iv) [ ] to disregard any amendments of the claims made under Article 19 and to consider them as reversed.

(v) [ ] to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

[X] The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)* except .....

(If the applicant does not wish to elect certain eligible States, the name(s) or country code(s) of those States must be indicated above.)

See Notes to the demand form

International application No.
PCT/NO93/00136

Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

				For International Preliminary Examining Authority use only: received	not received
1. amendments under Article 34					
description	:		sheets	☐	☐
claims	:	1	sheets	☐	☐
drawings	:		sheets	☐	☐
2. letter accompanying amendments under Article 34	:	1	sheets	☐	☐
3. copy of amendments under Article 19	:		sheets	☐	☐
4. copy of statement under Article 19	:		sheets	☐	☐
5. other *(specify)*: copy of search report	:	3	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ fee calculation sheet
5. ☐ other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

ONSAGERS PATENTKONTOR AS

Olav Robsahm

Olav Robsahm

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

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Demand received from IPEA on:

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Deres (Your) ref.	Vår (Our) ref.	Dato
	Fod 1 P93496 KM/HV	14 April 1994

Dear Sirs,

International Patent Application No. PCT/NO93/00136 -
Øystein Fodstad and Gunnar Kvalheim

./. In accordance with Article 34 we enclose a new set of claims in which claim 1 has been amended.

On page 28, line 4, we have added the following: "... solid tissues, with the exception of normal and malign haematopoietic cells in blood and bone marrow...". This amendment is performed in order to restrict the claim against the cited prior art in the International Search Report.

Yours faithfully,
Onsagers Patentkontor AS

Handled by: Kjell Myhre

Enclosure

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 Claims for deleting

CLAIMS

1. A ~~Improved~~ method for detecting specific target cells in cell suspensions of mixed cell populations and in fluid systems containing mixed cell populations, and in single cell suspensions prepared from solid tissues, with the exception of normal and malign haematopoietic cells in blood and bone marrow, characterized by comprising the following steps:

1.1. coating, by a per ce known procedure, paramagnetic particles or beads with either, a) antibodies, or antibody fragments directed against membrane structures specifically expressed on target-cells and not on non-target-cells in the cell mixture or;
b) antibodies, preferably polyclonal anti-mouse or monoclonal rat anti-mouse antibodies or anti-human antibodies, capable of binding to the Fc-portions of the said antibodies, directed against the membrane structures; and

1.2.1. mixing the target-cell-associating antibodies (murine or human) which is attached to the said particles or beads, or attached to the beads pre-coated with anti-mouse or antihuman antibodies recognizing the Fc-portions of the target-associating antibodies, with the cell suspension containing the target-cells, or,

1.2.2. mixing free target-cell-associating antibodies with the cell suspension containing the target cells and incubate this mixture for 5-10 min to 2 h, preferably 30 min, at a temperature between 0°C and 20°C, preferably 4°C under gentle rotation, and;

1.3. incubating the mixture of the cell suspension and target-associating antibodies attached to paramagnetic particles or beads (1.2.1), or paramagnetic particles or beads, precoated with anti-mouse or anti-human antibodies recognizing the Fc-portion of the target-associating antibodies, to the mixture of incubated free target associating antibody and cell suspension (1.2.2.), and incubating, for 5-10 min to 2 h,

preferably 30 min, at a temperature between 0°C and 25°C, preferably 4°C, under gentle rotation, and;

1.4.1. if the target cell population is contained in blood or bone marrow aspirates the hydrophobic forces associated with antibody-coated particles are reduced by pre-incubating the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, e.g. Tween 20™ in concentrations less than 0.1% for 30 min at 4°C, and/or

1.4.2. by incubating the cell suspensions, untreated or pretreated with formalin, alcohol or other fixatives, with other antibodies or antibody fragments binding to extracellular or intracellular molecules present in the target cells and the antibodies used are labeled in advance by peroxidase, alkaline phosphatase, or other enzymes permitting visualization of the binding by addition and incubation with relevant substrates, or

1.4.3. the antibody fragments are biotinylated and the binding visualized when adding the incubating with avidin complexed to peroxidase, alkaline phosphatase, or other enzymes, with addition and incubation with relevant substrates, or

1.5.1. subjecting the incubated paramagnetic particle-antibodies-cell mixture (1.3) to a magnetic field if the density of target-cells is low, or if the ratio of target cell/total cells in the cell mixture is low (≤ 1 %) and then examining and counting the stained or unstained particle-target-cell complexes in the cell suspension, using a micropscope and/or a suitable cell/particle counting device, or,

1.5.2. examining and counting the target-cells in the incubated mixture of paramagnetic particles, antibodies and cell mixture (1.3), or in the case when the antibodies or antibody fragments are conjugated to non-paramagnetic particles that can be visualized directly because of colour or through enzymatic activation, using a microscope and/or a suitable cell/particle

counting device if the ratio of target-cells/total cells in the cell suspension is adequate (> 1 %).

2. Method according to claim 1, characterized by directing the antibody or fragments thereof against the antigens in normal, living cells, such as liver hepatocytes, Kupffer cells and endothelial cells type 1 and 2 and Clara cells of the lung, endothelial cells of specific organs, pancreatic exocrine and endocrine cells, kidney tubule cells, bladder epithelial cells, brain glial and ependymal cells, bladder and prostate epithelial cells, ciliated cells of airways, different subpopulations of mucosal cells in the gastrointestinal tract, pituitary cells, and other endocrine cells in various hormone-producing organs.

3. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody which is reactive with antigens present on subpopulations of normal cells and oncogenic products expressed on the membrane of normal tissue cells.

4. Method according to one of the preceding claims, characterized by using as the said positive selecting antibody, an antibody which is directed against growth factor receptors on the membrane of normal cells, for example the EGF-receptor, PDGF (A and B) receptor, insuline receptors, insuline-like receptors transferrin receptor, NGF and FGF receptors.

5. Method according to one of the preceding claims, characterized by using an antibody directed against the group of integrins and other adhesion membrane molecules, and MDR proteins in normal cells.

6. Method according to one of the preceding claims, characterized by directing the antibody or fragments thereof against antigen or receptors in cells with abnormal developmental patterns, preferably such as primary and metastatic cancer cells.

7. Method according to one of the preceding claims, characterized by using as the said target-cell associating antibodies, antibodies of the IgG isotype, or $F(ab')_2$ or F(ab) fragments, or IgM, or fragments of IgM.

8. Method according to one of the preceding claims, characterized by preparing the mentioned cell suspension from mixed cell populations comprising mammalian tissues, for examples human bone marrow and peripheral blood, from pleural and peritoneal effusions, other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from solid tumors in normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissue, central nervous system, prostatic gland, skin and mucous membranes.

9. Method according to one of the preceding claims, characterized by that the antibody or antibody fragments is directed against groups of antigen determinants, such as those listed in the Table 1 of the specification.

10. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody or antibody fragment which is directed against growth factor receptors and oncogene products expressed on the membrane of malignant cells, for example insuline receptors, insuline-like receptors and FGF receptors in addition to those listed in Table 1 of the specification.

11. Method according to one of the preceding claims, characterized by using an antibody or antibody fragment directed against the group of integrins, other adhesion membrane molecules and MDR proteins in abnormal cells as listed in Table 1.

12. Method according to one of the preceding claims, characterized in that the used antibodies, antibody fragments

or combinations of these are directed to the antigen determinants as listed in Table 1 of the specification.

13. Method according to one of the preceding claims, characterized by using as the said antibody an antibody which is reactive with antigens present on abnormal cells, for example breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma and cancer cells of the gastrointestinal and genitourinary tract, and of the reticuloendothelial system, and/or target-cells associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune gastrointestinal, genitourinary, reticuloendothelial and other disorders.

14. Use of the detection method according to one of the preceding claims, for isolation of target-cells, whereby the complex of cells and the paramagnetic particles are exposed to a magnetic field and the resulting magnetically aggregated cells are further subjected to biological, biochemical and immunological examinations, including also characterisation of specific genes at the DNA, mRNA and protein level, including polymerase chain reaction (PCR) and reverse transcriptase PCR.

15. Use of the method for detection of specific target-cells according to one of the preceding claims, whereby it is established in vitro cell cultures from the separated paramagnetic particle-target-cell-complexes, and/or for inoculation into immunodeficient animals, preferably to establish human tumor xenografts in the said animals.

16. Kit for performing the method according to one of the preceding claims, characterized by that it comprises; 1, specific antibodies or antibody fragments directed to the antigen receptors on the wanted target-cells, where said antibody or antibody fragment is bound or can be bound to included paramagnetic particles, without removing their antigen-binding ability, and/or

2, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc portions of the target-cell associating antibodies, and specific free target-cell antibodies, and/or

3, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc-portions of the target-cell associating antibodies, bound to specific anti-target-cell antibodies, and/or

4, other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target cells, where said antibodies or antibody fragments are conjugated to biotin, peroxidase, alkaline phosphatase, or other enzymes, or where said antibodies or antibody fragments are bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
Fod 1 P 93496 Hv		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NO 93/00136	10/09/1993	14/09/1992
International Patent Classification (IPC) or national classification and IPC		
G01N33/53		
Applicant		
FODSTAD, Oystein et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of 22 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14/04/1994	06.12.94
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	F. Hallé Telephone No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.
PCT/NO93/00136

I. Basis of the report

1. This report has been drawn up on the basis of:

[ ] the international application as originally filed.

[x] the description, pages 14-18,22-27, as originally filed,
pages _____, filed with the demand,
pages 1-13,19-21, filed with the letter of 26.08.94,
pages _____, filed with the letter of _____,

[x] the claims, No. _____, as originally filed,
No. _____, as amended under Article 19,
No. _____, filed with the demand,
No. 1-16, filed with the letter of 26.08.94,
No. _____, filed with the letter of _____,

[ ] the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of: pages: _____
sheets of drawings/figures No.: _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed:

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-16	YES
	Claims	NO
Inventive Step (IS)	Claims 1-16	YES
	Claims	NO
Industrial Applicability (IA)	Claims 1-16	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

Having regard to the prior art documents cited in the International Search Report, the subject-matter claimed appears to meet the requirements of Article 33(2)(3)(4) PCT.

Indeed, the invention relates to a method for detecting target cells in mixed cell populations. Such a method as claimed is not anticipated by the prior art.

It is to be noted that the prior art document "WO-A-92 04961" (cited in the Search Report and referred to in the description) also refers to methods for magnetic separations. However, the methods disclosed in that document do not appear to be appropriate for examination of solid tissues contrary to present invention. Moreover, the magnetic system used in that prior art method substantially differs from that of present innvention. Therefore it seems unlikely that the present invention could be derived from the prior art without inventive skill.

KOPI til orientering

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT/NO 9 3 / 0 0 1 3 6
International Application No.

10 SEPT. 1993 (10.09.93)
International Filing Date

Styret for det industrielle rettsvern
PCT International Application
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) Fod 1 P93496 HV

Box No. I TITLE OF INVENTION Improved method for detection of specific target cells in specialized or mixed cell population and solutions containing mixed cell population

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Fodstad, Øystein
Frits Kiers v. 28

N-0383 OSLO
NORWAY

[X] This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: NO

State *(i.e. country)* of residence: NO

This person is applicant for the purposes of: [X] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Kvalheim, Gunnar
Åsstubben 13

N-0381 OSLO
NORWAY

This person is:
[ ] applicant only
[X] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: NO

State *(i.e. country)* of residence: NO

This person is applicant for the purposes of: [X] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
[ ] applicant only
[ ] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV · AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby [redacted] been appointed to act on behalf of the applicant(s) before the competent International Authorities as: [X] agent [redacted] [ ] common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Onsagers Patentkontor AS
P.O.Box 265 Sentrum

N-0103 OSLO
NORWAY

Telephone No.: +47 22 42 97 50

Facsimile No.: +47 22 33 65 94

Teleprinter No.: 77657 onpat n

[ ] Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

[X] EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

[X] OA OAPI Patent: Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Congo, Côte d'Ivoire, Gabon, Guinea, Mali, Mauritania, Niger, Senegal, Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

[X] AT Austria .....
[X] AU Australia .....
[X] BB Barbados
[X] BG Bulgaria .....
[X] BR Brazil .....
[X] BY Belarus
[X] CA Canada
[X] CH and LI Switzerland and Liechtenstein
[X] CZ Czech Republic .....
[X] DE Germany .....
[X] DK Denmark .....
[X] ES Spain .....
[X] FI Finland .....
[X] GB United Kingdom
[X] HU Hungary .....
[X] JP Japan .....
[X] KP Democratic People's Republic of Korea .....
.....
[X] KR Republic of Korea .....
[X] KZ Kazakhstan .....
[X] LK Sri Lanka
[X] LU Luxembourg .....
[X] MG Madagascar
[X] MN Mongolia .....
[X] MW Malawi .....
[X] NL Netherlands
[X] NO Norway
[X] NZ New Zealand .....
[X] PL Poland .....
[X] PT Portugal .....
[X] RO Romania
[X] RU Russian Federation .....
[X] SD Sudan
[X] SE Sweden
[X] SK Slovakia .....
[X] UA Ukraine
[X] US United States of America .....
.....
[X] VN Viet Nam .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

[ ] .....
[ ] .....
[ ] .....
[ ] .....

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____.
The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
(1) NO	14 September 1992 (14.09.92)	PCT/NO92/00151	RO/NO
(2)			
(3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to transmit to the International Bureau a certified copy of the earlier application(s) identified above at item(s) : (1)

Box No. VII EARLIER SEARCH

Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	:	3 sheets
2. description	:	27 sheets
3. claims	:	6 sheets
4. abstract	:	1 sheets
5. drawings	:	0 sheets
Total	:	37 sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *(specify)*:
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing
8. ☒ other *(specify)*: copy of int. search report in priority appln.

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

ONSAGERS PATENTKONTOR AS

Olav Robsahm

Olav Robsahm

For receiving Office use only

1. Date of actual receipt of the purported international application: 10 SEPT. 1993 (10.09.93)
2. Drawings: ☐ received : ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA / SE
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

NOTED, PRIORITY DOC. WILL FOLLOW. RO/NO

PATENT COOPERATION TREATY

ONSAGERS
13 JUNI 1994
PATENTKONTOR

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
ONSAGERS Patentkontor AS
P.O.Box 265 Sentrum
0103 OSLO 1
NORVEGE

WRITTEN OPINION

(PCT Rule 66)

Frist notert:
10/8 Sm

Date of mailing *(day/month/year)*	10. 06. 94
Applicant's or agent's file reference: Fod 1 P 93496 Hv	REPLY DUE within 3 months/~~days~~ from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NO 93/00136	10/09/1993	14/09/1992

International Patent Classification (IPC) or both national classification and IPC
G01N33/53

Applicant
FODSTAD, Oystein et al

1. This written opinion is the FIRST (first, etc.) drawn up by this International Preliminary Examining Authority.
2. This report contains indications and corresponding pages relating to the following items:
   - I [X] Basis of the opinion
   - II [ ] Priority
   - III [ ] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [X] Certain observations on the international application
3. The applicant is hereby **invited to reply** to this opinion.
   - **When?** See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).
   - **How?** By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.
   - **Also** For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4*bis*. For an informal communication with the examiner, see Rule 66.6.

   If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.
4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 14/01/1995.

Name and mailing address of the IPEA/
European Patent Office
D-80298 Munich
Tel. (+49-89) 2399-0, Tx: 523656 epmu d
Fax: (+49-89) 2399-4465

Authorized officer
Examiner F. Hallé

Formalities officer
(incl. extension of time limits)
Telephone No. 2399-8161 Waltraud Hebert

Intern. application No.

WRITTEN OPINION PCT/NO93/00136

I. Basis of the opinion

1. This opinion has been drawn up on the basis of:

[ ] the international application as originally filed.

[x] the description, pages 1-27, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,

[x] the claims, No. _____, as originally filed,
No. _____, as amended under Article 19,
No. _____, filed with the demand,
No. 1-16, filed with the letter of 14.04.94,

[ ] the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,

2. The amendments have resulted in the cancellation of: pages: _____
sheets of drawings/figures No.: _____.

3. [ ] This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed:

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-16 (YES) Claims
Inventive Step (IS)	Claims 1-16 (NO) Claims
Industrial Applicability (IA)	Claims 1-16 (YES) Claims

2. CITATIONS AND EXPLANATIONS

1. The following document (D) is referred to in this communication; the numbering will be adhered to in the rest of the procedure:

D1: WO-A-92 04961

2. The present application satisfies the criterion set forth in Article 33(2) PCT because the subject-matter of Claims 1-16 is new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

3.1 However, it seems that the present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter claimed does not involve an inventive step (Rule 65(1)(2) PCT). Indeed, the object of the present invention is to detect and examine particular target cells in cell suspensions of mixed cell populations, without unspecific binding to normal cells. The solution proposed is to use an insoluble magnetic support, coated with specific antibodies to cell mem-

brane antigens, to form a complex between the traget cells and the magnetic insoluble support. The method is such that a later cleavage between the insoluble support and the target cells is not necessary.

3.2 However, in the relevant prior art D1 it is also mentioned that a separate resuspension of the target cells is avoided and that analytical procedures as quantitative determinations may be performed on the magnetically immobilized colloid (see D1, in particular, page 23). Therefore, the present invention appears to be obvious to the skilled person.

3.3 The Applicant is requested to show advantageous features of the process claimed in the present application over the prior art process which could substantiate inventivity.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1. The subject-matter of Claim 1 should be identified as "A method for..." instead of "Improved method for...".

2. To meet the requirements of Rule 5.1(a)(ii) PCT, the document D1 should be identified in the description and the relevant background art disclosed therein should be briefly discussed.

3. The PCT application numbers mentioned in the description should be replaced by the corresponding publicly accessible publication numbers.

4. The terms Tween (page 11, claim 1.4.1) and Dynabeads (pages 19, 21) appear to be registered trade marks and should be identified as such.

5. The Applicant is requested to file amendments by way of replacement pages. He should also take into account the requirements of Rule 66.8 PCT. In particular, fair copies of the amendments should preferably be filed in triplicate.

   In order to expedite further examination you are requested to indicate with your reply the locations in the application as originally filed of the passages forming a basis for the amendments.

   The attention of the Applicant is drawn to the fact that the application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed, Article 34(2)(b) PCT.

Any information the Applicant may wish to submit concerning the subject-matter of the invention, for example further details of its advantages or of the problem it solves, and for which there is no basis in the application as filed, should be confined to the letter of reply rather than be incorporated into the application, Article 34(2)(b) PCT.

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 33/53, C12Q 1/00
C12N 5/00

A1

(11) International Publication Number: WO 94/07139

(43) International Publication Date: 31 March 1994 (31.03.94)

(21) International Application Number: PCT/NO93/00136

(22) International Filing Date: 10 September 1993 (10.09.93)

(30) Priority data:
PCT/NO92/00151 14 September 1992 (14.09.92) WO

(34) Countries for which the regional or international application was filed: NO et al.

(71)(72) Applicants and Inventors: FODSTAD, Øystein [NO/NO]; Frits Kiers v. 28, N-0383 Oslo (NO). KVALHEIM, Gunnar [NO/NO]; Åsstubben 13, N-0381 Oslo (NO).

(74) Agent: ONSAGERS PATENTKONTOR AS; P.O. Box 265 Sentrum, N-0103 Oslo (NO).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: IMPROVED METHOD FOR DETECTION OF SPECIFIC TARGET CELLS IN SPECIALIZED OR MIXED CELL POPULATION AND SOLUTIONS CONTAINING MIXED CELL POPULATIONS

(57) Abstract

The invention relates to a method for detecting specific target-cells in a simple and time saving way, using paramagnetic particles, antibodies recognizing the Fc portions of target-cell associating antibodies and target-cell associating antibodies directed to specific antigen determinants in the target-cell membranes. Incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method. The method can further be used for isolation of the target-cells by magnetic field application and a kit for performing the method according to the invention is described.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
Fod 1 P 93496 Hv		
International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/NO 93/00136	10/09/1993	14/09/1992
International Patent Classification (IPC) or national classification and IPC		
G01N33/53		
Applicant		
FODSTAD, Oystein et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of 22 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14/04/1994	06.12.94
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	F. Hallé Telephone No.

Intern. application No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

PCT/NO93/00136

I. Basis of the report

1. This report has been drawn up on the basis of:

[ ] the international application as originally filed.

[x] the description, pages 14-18, 22-27, as originally filed,
pages _____, filed with the demand,
pages 1-13, 19-21, filed with the letter of 26.08.94,
pages _____, filed with the letter of _____,

[x] the claims, No. _____, as originally filed,
No. _____, as amended under Article 19,
No. _____, filed with the demand,
No. 1-16, filed with the letter of 26.08.94,
No. _____, filed with the letter of _____,

[ ] the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of: pages: _____
sheets of drawings/figures No.: _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed:

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-16	YES
	Claims	NO
Inventive Step (IS)	Claims 1-16	YES
	Claims	NO
Industrial Applicability (IA)	Claims 1-16	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

Having regard to the prior art documents cited in the International Search Report, the subject-matter claimed appears to meet the requirements of Article 33(2)(3)(4) PCT.

Indeed, the invention relates to a method for detecting target cells in mixed cell populations. Such a method as claimed is not anticipated by the prior art.

It is to be noted that the prior art document "WO-A-92 04961" (cited in the Search Report and referred to in the description) also refers to methods for magnetic separations. However, the methods disclosed in that document do not appear to be appropriate for examination of solid tissues contrary to present invention. Moreover, the magnetic system used in that prior art method substantially differs from that of present innvention. Therefore it seems unlikely that the present invention could be derived from the prior art without inventive skill.

[redacted]

Improved method for detection of specific target cells in specialized or mixed cell population and solutions containing mixed cell populations

The present invention relates to an immunomagnetic method for detection of specific target cells in cell populations and solutions of cell populations. The invention also relates to a kit for performing the method in different cell populations.

In biology, biochemistry and adjacent fields it is considerable need for methods in which chemical entities are linked together. Such methods have an increasing importance in research regarding both normal and abnormal cell populations. Especially when solid supports are used cells can be immobilized, detected and isolated and purified. Furthermore, the cell content may be examined using a range of sofisticated methods. It is also of importance to be able to isolate the cells in viable forms.

Affinity binding is a sofisticated way of linking chemical/biochemical entities together. In such a method a pair of binding partners, which for example are attached to the substances to be linked, bind to each other when brought in contact. One of the binding partners in such a linkage may be represented by a molecule on the cell surface. Several such binding partner systems are known, such as antigen- antibody, enzyme- receptor, ligand- receptor interactions on cells and biotin- avidin binding, of which antigen-antibody binding is most frequently used. A hapten/anti-hapten binding pair method has also recently been suggested (WO 91/01368).

When such methods are used for isolation of specific cells, which are the subject for further various examinations, it is necessary to reverse the linkage without producing destructive effects on the binding partners, which ideally should recover their function upon returning to the original conditions. This is not always the case, although it is proposed a method for

adequately cleaving antigen/anti-antigen and hapten/anti-hapten linkages (WO 91/15766, WO 91/01368).

Methods are known in which one of the binding partners is attached to an insoluble support, such as paramagnetic particles, and by which isolation of target cells in a mixed cell population is performed as negative isolation or positive isolation. In a negative isolation procedure the unwanted cells can be removed from the cell preparation by incubating the cells with antibody-coated particles, specific for the unwanted cells. Following the incubation the cell/antibody/particle-complex can be removed using the particles, leaving the wanted target cells behind. This result is often not satisfactory, since the wanted cells are left in the cell population, more or less purified, and also since the intention of the isolation procedure is to examine and/or perform further studies on the specific target cells. Attempts have been made to use particles for positive isolation, in which the wanted target cells are removed from the mixed cell population. These procedures have, however, been directed to certain target cells and are not suited for all target cell systems. A positive isolation procedure involving the hapten/anti-hapten linkage system has recently been proposed (WO 91/01368) and also a method for isolating haemopoietic progenitor cells from bone marrow (WO 91/09938). The latter is directed to use a combination of positive and negative selection for the purpose of isolating and possibly growing specific cells, i.e. haematopoietic progenitor cells, in the bone marrow, and is dependent upon removal of the particles.

WO 91/01368 relates to a method of connecting target cells to an insoluble support by using the abilities of hapten, anti-hapten antibodies and anti-cell antibodies to bind to each other, thus constructing a linkage between the insoluble support, i.e. particle, and the target cell, consisting at least of hapten and anti-hapten antibody or combinations of hapten and anti-hapten antibodies and anti-anti-hapten antibodies or secondary anti-cell antibodies. The later

cleavage of the complex is performed by again exposing it to hapten or hapten analogue. Thus the constructed link always consists of hapten in addition to 1 or more elements. The method is directed to unspecified target cells and is directed to isolation of target cells and release of the insoluble support.

There is a need for a simple linkage to connect a target cell to an insoluble support, which do not involve compounds of a rather unspecified haptene-group, and which is directed to detection of specific target cells, with a minimum of unspecific cell association and which render unnecessary a later cleavage between the insoluble support and the specific target cell.

WO 92/04961 comprises a method and a complicated equipment to separate cells or different molecules from a non-magnetic test medium by using collodial magnetic particles. In this method small (sub micron) particles are used because it is necessary to avoid precipitation of the particles in the solution and their method necessitates a complicated separation system, in which magnetic intensifying means is immersed in the test medium. This may have adverse effects on the cells.

Thus the object of the present invention is to detect for diagnostic purposes specific target cells when used in a blood and bone marrow, without the problem with unspecific binding to normal cells. It represents a sensitive detection method for a variety of cell types, such that a high number of cells can be readily screened in the microscope and the procedure is rapid and simple. Furthermore, the present method can be used for isolation of cells for biochemical, biological and immunological examination, and for studying of specific genes at the nucleotide or protein level, in addition to culturing the cells, without the need for cleaving the cell-particles complex. A further object of the invention is to provide a kit for performing the method as characterized in the claims.

The intensions of the inventions are obtained by the method and kit characterized in the enclosed claims.

The method for immunomagnetic detection of target cells in a mixed cell population and physiological solutions containing cell populations is suitable for detection, but may also be used in positive isolation of specific types of both normal cells and patogenic cells. The method creates a linkage between a specific target cell and an insoluble support, such as paramagnetic particles , which consists of one or two elements. The particle is either coated with an anti-cell antibody of murine or human origin, directed to the specific antigen determinants in the membranes of the wanted target-cells, or the particles are coated with a polyclonal anti-mouse or anti-human antibody capable of binding to the Fc-portions of the specific anti-cell antibody directed to the antigen determinants in the target-cell membranes. Instead of using the polyclonal anti-mouse/anti-human antibody for coating the particles, a monoclonal rat anti-mouse/anti-human antibody may be used. This last antibody, due partly to its monoclonal origin, may provide a more specific binding to the anti-cell antibody and reduce the risk for possible cross-reactions with other cells in solutions, such as blood. Furthermore, incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method.

In the following a more detailed disclosure of the method is presented, using cancer cells as the target-cells for detection and possible isolation. The method is, however, not limited to cancer cells and the disclosure shall not limit the method to this particular field of use, since the method is suitable within a range of cytological research areas.

In the management of cancer patients, the staging of the disease with regards to whether it is localized or if

metastatic spread has occurred to other tissues, is of utmost importance for the choice of therapeutic alternative for the individual patient. Malignant cells spread by direct invasion into the surrounding tissue, through the lymphatics or by the distribution of tumor cells in the blood to distant organs, including the bone marrow and the central nervous system and the cerebrospinal fluid.

Detection of metastatic tumor cells has, until recently, relied on morphological methods using light and electron microscopy on biopsied tumor specimens, on smears of bone marrow and peripheral blood, and on slides prepared after cytosentrifugation of various body fluids. Since the advent of monoclonal antibodies recognising antigens predominantly expressed on the surface of different types of malignant cells, the identification of metastatic cells has, to an increasing extent, also involved immunocytochemistry and immunofluorescence. Thus, slides prepared from biopsied tumors or cytosentrifugates have been treated with monoclonal antibodies, and the binding of these to the tumor cells is visualized colorimetrically or by fluorescence. The latter method requires the use of a fluorescence microscope, alternatively preparing a cellsuspension an use a flow cytometer.

The previous methods suffer from limited sensitivity and/or specificity, and is usually laborious and time consuming, also requiring a high degree of expertise. Flowcytometric examiniations also involve expensive equipment.

The morphological methods for the detection of tumor cells in blood and bone marrow are much less sensitive than methods involving immunocytochemistry and immunofluorescence (Beiske et al., Am. J. Pathology 141 (3), September 1992). Also the latter methods are, however, inadequate in cases where the tumor cells represent less than 1 % of the total number of nucleated cells. Flow cytometry may provide better sensitivity than the methods involving the use of a microscope, but requires the

availability of a high number of cells, and also involves several technical difficulties. Thus, aggregation of cells may cause problems, and the method does not provide possibilities to distinguish between labeled tumor cells and unspecifically fluorescing normal cells.

The invention allows for a very sensitive detection of, for example, metastatic tumor cells, since a high number of cells can readily be screened in the microscope and the attached magnetic beads are easily recognisable. The monoclonal antibodies used bind with sufficient specificity to, for example, tumor cells and not to other cells than the target-cells present in mixed cell suspensions, like blood, bone marrow, and in other tumor manifestations, such that all cells with attached beads represent the target-cells. In addition, the procedure is rapid and simple, and can be performed by any investigator with access to a conventional microscope.

The novel method involves the binding of monoclonal antibodies, e.g. of murine or human origin, that specifically recognize antigens present on tumor cells, and not on the normal cells in question, or for other purposes to specified subpopulations of normal cells, to paramagnetic particles, either directly or to beads first covered with antibodies specifically recognizing the respective antibodies, or the Fc-portion of IgG antibodies, that bind to the tumor cells. The cell binding antibodies may be of the IgG or IgM type or being a fragment of ab IgG or IgM. Examples of used anti-target-cell antibodies may be those directed against groups of antigen determinants, for example CD56/NCAM antigen (MOC-1), Cluster 2 epithelial antigen (MOC-31), Cluster 2 (MW 40kD) antigen (NrLu10) (Myklebust et al. Br. J. Cancer Suppl. 63, 49-53, 1991), HMW-melanoma-associated antigen (9.2, 27) (Morgan et al., Hybridoma, 1, 27-36, 1981), 80kD, Sarcoma-associated antigen (TP1 & TP3) (Cancer Res. 48, 5302-5309, 1988), mucin antigens (Diel et al., Breast Cancer Res. Treatm., 1991), or EGF-receptor antigen (425.3) (Merck), in addition to the anti-pan-human antibody (Bruland et al., unpublished), which is suitable for detecting human cells

among animal cells. The 425.3 antibody is directed towards antigens in both normal and malignant cells. Antibodies can furthermore be directed against growth factor receptors, for example EGF-receptor, PDGF (A and B) receptor, insuline receptor, insuline-like receptor, transferrin receptor, NGF and FGF receptors, group of integrins, other adhesion membrane molecules and MDR proteins in both normal cells and abnormal cells, and antigens present on subpopulations of normal cells, in addition to oncogenic products, expressed on the membranes of normal and malignant cells and on malignant cells alone, for example Neu/erb B2/HER2. As for the malignant cells, these may be breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma, cancer cells of the gastrointestinal tract and the reticuloendothelial system, or the target-cells may be associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune, gastrointestial, genitourinary, reticuloendothelial and other disorders. Furthermore, the malignant cell population may be located in bone marrow, peripheral blood, come from pleural and peritoneal effusions and other body fluid compartments, such as urine, cerebrospinal fluid, semen, lymph or from solid tumors in normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissue, the central nervous system, prostatic gland, skin and mucous membranes. A more complete list of the antigen determinants and the corresponding antibodies or antibody fragments used in the present improved method is presented in Table 1.

The method comprises attachment of the antibodies directly to the paramagnetic particles, or the attachment can take place by attachment to surface-bound antibodies, such as polyclonal anti-mouse antibodies, monoclonal rat anti-mouse antibodies or monoclonal anti-human antibodies, specifically recognizing the Fc-portion of the said individual antibodies. The antibody-coated paramagnetic beads are then mixed with the suspension of cells to be examined and incubated for 5-10 min to 2 h, preferably for 30 min at 0-25°C, preferably at 4°C, under gentle rotation. The present method may also be performed in a

changed order of steps, in that the free target-cell antibodies are added to the cell suspension, incubated for 5-10 min to 2h, preferably 30 min, at 0-20°C, preferably 4°C, under gentle rotation. The paramagnetic particles, precoated with anti-mouse or anti-human antibodies are then added to the incubated cell suspension, as described above, and the resulting suspension subjected to a further incubation of 5-10 min to 2h, preferably 30 min, at 0-25°C, preferably 4°C under gentle agitation. Samples of the cell suspension are then transferred to a cell counting device, and the fraction of cells with attached beads relative to the total number of cells is determined under light microscopy. The number of antibody-coated beads added to the cell suspension should be between 0.5-10 times the number of target cells. When this number is unknown, the amount of coated beads added should be 1-10 % of the total number of cells.

For specific purposes, and in the cases where the density of the target-cells is low, for example malignant cells, or the target-cells represent a very low fraction of the total number of cells (≤ 1%), the target cells can be positively separated from non-target cells in a magnetic field. The isolated target cells, can then be enumerated microscopically and the fraction of target cells relative to the total number of cells in the initial cell suspension can be calculated. Moreover, the target-cells may be characterized for the presence of specific biochemical and biological features. Of particular importance will be the use of such cells for studies in molecular biology. In contrast to the above cited methods of the prior art, the present method allows studies and growth of the target-cells without performing a cleavage of the paramagnetic particle-target cell linkage. For several purposes it is of interest to examine specific genes in a pure population of target cells at the DNA, mRNA and protein level, both in tumor biopsies as well as in tumor cells present in blood, bone marrow and other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from otherwise normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissues, central

nervous system, prostatic gland, skin and mucous membranes, and in other areas of cytological research activity.

With the methods of prior art, signals obtained on Southern, Northern and Western blots represent the normal cells as well as the tumor cells in the biopsy. If a single cell suspension is first prepared from the tumor material, and the tumor cells are then positively immunomagnetically detected and separated, any gene studies performed on this material would represent the target-cells only. This also relates to for example malignant cells present in mammalian tissues, for example in bone marrow, peripheral blood, pleural and peritoneal effusions, and other body fluids, for example urine, cerebrospinal fluid, semen and lymph. Studies involving polymerase chain reaction (PCR) methodology will also gain in specificity and reliability when performed on pure tumor cell populations obtained by the new method.

The application of the new method steps may differ depending on type of tissues to be examined.

a) Tissue from solid or needle tumor biopsies is prepared mechanically or with mild enzymatic treatment into a single cell suspension, to which the primary, specific antibodies or antibody fragments are added directly or after washing the cell suspension with phosphate buffered saline or culture medium with or without serum, such as fetal calf serum, bovine, horse, pig, goat or human serum.

b) If the material is a sample of pleural or ascitic effusion, cerebrospinal fluid, urine, lymph or body fluids such as effusions in the joints of patients with various forms of arthritis, the specific antibodies or antibody fragments are either added to the samples directly, or after centrifugation with or without washings before or after the cells in the samples are spun down and brought back into suspension.

c) If the material consists of blood or bone marrow aspirate, the mononuclear cell fraction is isolated by gradient sentrifugation on e.g. Lymphoprep before washing, resuspension, and addition of the appropriate antibodies or antibody fragments.

The procedure conditions for a) and b) are established, as exemplified by results obtained in successful experiments as those described below.

For c) the results have been found to be influenced by a high number of factors which have been examined in detail. Among these are antibody concentration, the ratio of the number of paramagnetic particles versus number of cells, incubation times and volumes, type of incubation medium, and the pH level. The particle to mononuclear cell ratio in all experiments should be in the range of 0.5/1 - 2/1, depending on the binding affinity of the primary specific antibodies or fragments.

A major problem has been unspecific attachment to normal blood or bone marrow cells of particles coated with either sheep or rat anti-mouse antibodies alone, or in addition with the specific antibodies. Experiments have shown that the unspecific binding is equally high without the presence of the specific antibodies, indicating that the problem is not caused by cross-reactivity of the targeting antibodies to normal cells. The possibility that the less than optimal specificity could be caused by ionic binding has been ruled out. Another possibility was that subpopulations of normal cells of the B-lineage might adhere to the particle-antibody complexes. However, immunomagnetic removal of B-cells from the cell suspension before adding the specific antibodies/antibody-particle complexes did not improve the specificity of the latter.

The problem with the procedure used on isolated mononuclear fractions of bone marrow and peripheral bllod, that some non-target cells might also bind paramagnetic particles, has been circumvented or overcome. Thus with sheep-anti-mouse antibody

coated particles alone or with specific antibodies the number of particles unspecifically attached to a low fraction mononuclear blood or bone marrow cells was reduced from an average of 10 to about 1 and in paralell the fraction of normal cells with particles decreased from 1-2% to 0.5-1% or less.

Evidence has been obtained that the problem may be caused by hydrophobic forces associated with the antibodies bound to the paramagnetic particles. Methods for reducing this hydrophobicity is thus claimed. One such method is preincubation of the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, for example Tween 20(TM) in concentrations of less than 0.1% for 30 minutes at 4°C. When possible selection of the target cells is warrented, the cell suspension should contain a low concentration of the detergent, e.g. 0.01% of Tween 20(TM). In several experiments this procedure has almost eliminated or dramatically reduced the problem of unspecific binding seen with the mononuclear cell fractions from blood or bone marrow.

The other improvement which, if found warrantied, may be used together with the detergent step as follows:

After incubation of the cell suspension with the primary antibodies or antibody fragments and the antibody-coated paramagnetic particles as described in previously, the cell suspension is incubated with a second set of antibodies or antibody fragments directed against other extracellular or against intracellular determinants of the target cells, with our without pretreatment with cell fixatives such as formaldehyde or alcohols. These antibodies or their fragments should have been prelabeled by fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or enzymes like peroxidase and alkaline phosphatase, allowing visualization by per se known methods in the microscope and/or a suitable counting device.

The target cells will both be visualized with the latter method and have bound particles to their surface, and can thus be enumerated.

To simplify the distinction between non-target and target cells, the cell suspension can before the second visualization step either be subjected to cytospin centrifugation or portions of the suspension are attached to caoted glass slides on which the particle-bound cells will be spread out in a thin layer, facilitating the recognition of the double-"stained" cells.

For use in the new procedure, kits will contain for example precoated paramagnetic particles prepared for each monoclonal antibody. In another embodiment the kits contain paramagnetic particles pre-coated with IgG isotype specific anti-mouse or anti-human antibody as one part of it, and different target cell-associated, for example tumor cell, antibodies as another part. In a third embodiment the kit contains paramagnetic particles precoated with specific anti-Fc antibodies, such as polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-mouse, or anti-human antibodies, capable of binding to the Fc-portion the target-cell associating antibodies, bound to specific anti-target-cell antibodies. In a further embodiment the kit contains other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target-cells, where said antibodies or antibody fragments are conjugated to peroxidase, alkaline phosphatase, or other enzymes, together with relevant substrates to such enzymes, or where said antibody or antibody fragment is bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

The present method will in the following be illustrated by model experiments, examples of the usefulness of the new method and examples of practical applications. These examples shall not be regarded as in any way limiting the invention.

Model experiments:

1. Binding of antibody-bead complexes to tumor cell lines with the new procedure:

To determine antibody concentrations and optimal conditions for the binding of antibody-paramagnetic particle complexes to tumor cells, a large panel of cancer cell lines was used. The paramagnetic beads were bound to the cells, either by coating the specific antibodies to sheep-anti-mouse antibody (SAM)-coated paramagnetic particles, or by first incubating the cells with the specific antibodies, washing, followed by a second incubation with SAM-coated particles. The results of these experiments are given in Tables 2a and 2b, in which + indicates binding of several beads to all cells, (+) indicates either a lower number of beads bound to each cell, or that not all the tumor cells had beads attached to their surface, whereas - reflects no binding, and (-) indicates very weak binding.

2. For detection of tumor cells in the mononuclear fraction of bone marrow or peripheral blood, model experiments were performed where specific antibodies and SAM-coated paramagnetic particles were added either to such mononuclear cells or to a cell suspension where a different number of cancer cells from in vitro cultivated cell lines were added to said mononuclear cells. In some experiments, either the mononuclear cells, or the malignant cells were prestained with a fluorescent dye, to be able to distinguish beteween the two types of cells. In all experiments, non-binding primary antibodies, and/or sheep-anti-mouse antibody-coated beads were used separately as controls.

several types of cancer. The detection of binding of such antibodies to respective antibodies can therefore be used to obtain information of high prognostic value. Among such antigens are a high number of adhesion molecules, carbohydrate antigens, glycolipids, growth factor receptors and carcinoma markers listed below. We have, with the new procedure identified the binding of particle-antibody complexes to CD44-variants, E-cadherin, Le^Y, CEA, EGF-r, transferrin receptor, MUC-1 epitope, LUBCRU-G7 epitope, prostate cancer antigen, UJ13A epitope, β_2-microglobulin, HLA-antigens, and apoptosis receptor.

Example 3

Two litres of pleural diffusion from a patient supposed to suffer from malignant melanoma was obtained. After centrifugation, the cells were suspended in a volume of 2 ml of RPMI with a 10% fetal calf serum, incubated with 9.2.27 anti-melanoma antibody (10 µg/ml) at 4°C for 30 min, washed and again incubated with Dynabeads(TM) SAM M450/IgG2A at 4°C for 30 min. The cell suspension was then examined under a microscope for determining the fraction of cells with paramagnetic cells attached to their surface. The diagnosis of malignant melanoma was confirmed, as about 10% of the cells had a significant number of particles rosettes.

Example 4

Biopsied tissue was obtained from a subcutaneous tumor in a case with clinical indications of either small cell lung cancer or a malignant melanoma. A single cell suspension was prepared from the biopsy, divided in 2 fractions, one incubated with the 9.2.27 anti-melanoma antibody, and the other with MOC-31 anti-carcinoma antibody (both at 10 µg/ml). The incubation was similar to that used in the example above. None of the cells incubated with the melanoma antibody bound any beads, whereas all tumor cells incubated with MOC-31 were positive.

Example 5

Biopsied tissue from a patient suspected to have malignant melanoma was examined by preparing single cell suspension, incubating with 9.2.27 anti-melanoma antibody, and then following the procedure as above. Most of the cells were positive with a high number of particle-rosettes attached to their membranes.

Example 6

A pleural effusion from a breast cancer patient was studied to examine whether tumor cells could be detected in the fluid. One litre of the fluid was centrifuged, the cells resuspended, and in separate vials incubate with each of 3 different anti-carcinoma antibodies (MOC-31, 2E11, 12H12). After completing the procedure as in the previous example, it was found that most of the cells bound to antibody-coated particles in all 3 cases.

Example 7

A bone marrow suspension obtained from a breast cancer patient was studied to examine whether micrometastic tumor cells could be present. After the preparation of mononuclear cells, these were incubated with the same 3 anti-carcinoma antibodies used in the example above, but in this case the antibodies were first attached to Dynabeads(TM) SAM IgG paramagneteic particles. After 1 incubation with these directly coated particles, the cell suspension was examined in the microscope, and a high number of cells were found positive with a number of particle-rosettes attached to their membrane.

Similar experiments have been performed in a number of pleural or ascitic effusion and bone marrow from patients with breast cancer.

Example 8

T47D human breast carcinoma cells were incubated for varying lenghts of time with Hoechst fluoresence dye, and the viability of the labeled cells was checked. Varying numbers of labeled breast carcinoma cells were then added to 1 x 10^6 bone marrow

cells obtained from healthy volunteers. In different experiments, different concentrations of paramagnetic, monodisperse particles (Dynabeads(TM) P450) coated with individual anticarcinoma antibodies (NrLu10, MOC31, or 12H12) were added. After incubation for 30 min on ice, samples of the different test tubes were examined in a counting chamber under light and fluorescence microscopy. When the ratio of tumor cells/total nucleated cells was low, the cell suspension was subjected to a magnetic field and the cells with particles attached were isolated before examined in the microscope. It was found that at an optimal ratio of 1-10 paramagnetic beads per tumor cell in the cell mixture, all the tumor cells had from 2-15 beads attached to their surface. The sensitivity of the detection method was close to one target-cell per 10^4 nucleated cells.

In control experiments with labeled tumor cells using antibodies known to have some cross-reativity to normal cells, this cross-reactivity was confirmed with the antibody-coated paramagnetic particles. In experiments with beads without tumor-associated antibody coating, none of the target cells bound any beads.

Similar experiments have been performed both with other breast cancer lines and a small cell lung cancer cell line. Similar sensitivity and specificity were obtained in these experiments.

Example 9

Pleural and ascites fluid from patients with breast cancer and ovarian carcinoma were sentrifuged, the same coated paramagnetic particles used in Example 1 were added, incubated and concentrated in a magnetic field before the suspension was examined under light microscopy. Typically, cells that had the clear morphological features of tumor cells had beads attached, whereas none of the few normal cells bound the antibody-coated beads. In two cases with pleural effusion, an independent morphological examination did not reveal the presence of any tumor cells, whereas a significant number malignant cells were detected by the use of anibody-coated beads. In some cases,

CLAIMS

1. A method for detecting specific target cells in cell suspensions of mixed cell populations and in fluid systems containing mixed cell populations, and in single cell suspensions prepared from solid tissues, characterized by comprising the following steps:

1.1. coating, by a per ce known procedure, paramagnetic particles or beads with either, a) antibodies, or antibody fragments directed against membrane structures specifically expressed on target-cells and not on non-target-cells in the cell mixture or;
b) antibodies, preferably polyclonal anti-mouse or monoclonal rat anti-mouse antibodies or anti-human antibodies, capable of binding to the Fc-portions of the said antibodies, directed against the membrane structures; and

1.2.1. mixing the target-cell-associating antibodies (murine or human) which is attached to the said particles or beads, or attached to the beads pre-coated with anti-mouse or antihuman antibodies recognizing the Fc-portions of the target-associating antibodies, with the cell suspension containing the target-cells, or,

1.2.2. mixing free target-cell-associating antibodies with the cell suspension containing the target cells and incubate this mixture for 5-10 min to 2 h, preferably 30 min, at a temperature between 0°C and 20°C, preferably 4°C under gentle rotation, and;

1.3. incubating the mixture of the cell suspension and target-associating antibodies attached to paramagnetic particles or beads (1.2.1), or paramagnetic particles or beads, precoated with anti-mouse or anti-human antibodies recognizing the Fc-portion of the target-associating antibodies, to the mixture of incubated free target associating antibody and cell suspension (1.2.2.), and incubating, for 5-10 min to 2 h,

preferably 30 min, at a temperature between 0°C and 25°C, preferably 4°C, under gentle rotation, and;

1.4.1. if the target cell population is contained in blood or bone marrow aspirates the hydrophobic forces associated with antibody-coated particles are reduced by pre-incubating the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, e.g. Tween 20(TM) in concentrations less than 0.1% for 30 min at 4°C, and/or

1.4.2. by incubating the cell suspensions, untreated or pretreated with formalin, alcohol or other fixatives, with other antibodies or antibody fragments binding to extracellular or intracellular molecules present in the target cells and the antibodies used are labeled in advance by peroxidase, alkaline phosphatase, or other enzymes permitting visualization of the binding by addition and incubation with relevant substrates, or

1.4.3. the antibody fragments are biotinylated and the binding visualized when adding the incubating with avidin complexed to peroxidase, alkaline phosphatase, or other enzymes, with addition and incubation with relevant substrates, or

1.5.1. subjecting the incubated paramagnetic particle-antibodies-cell mixture (1.3) to a magnetic field if the density of target-cells is low, or if the ratio of target cell/total cells in the cell mixture is low (≤ 1 %) and then examining and counting the stained or unstained particle-target-cell complexes in the cell suspension, using a micropscope and/or a suitable cell/particle counting device, or,

1.5.2. examining and counting the target-cells in the incubated mixture of paramagnetic particles, antibodies and cell mixture (1.3), or in the case when the antibodies or antibody fragments are conjugated to non-paramagnetic particles that can be visualized directly because of colour or through enzymatic activation, using a microscope and/or a suitable cell/particle

counting device [redacted] the ratio of target-cells [redacted] otal cells in the cell suspension is adequate (> 1 %).

2. Method according to claim 1, characterized by directing the antibody or fragments thereof against the antigens in normal, living cells, such as liver hepatocytes, Kupffer cells and endothelial cells type 1 and 2 and Clara cells of the lung, endothelial cells of specific organs, pancreatic exocrine and endocrine cells, kidney tubule cells, bladder epithelial cells, brain glial and ependymal cells., bladder and prostate epithelial cells, ciliated cells of airways, different subpopulations of mucosal cells in the gastrointestinal tract, pituitary cells, and other endocrine cells in various hormone-producing organs.

3. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody which is reactive with antigens present on subpopulations of normal cells and oncogenic products expressed on the membrane of normal tissue cells.

4. Method according to one of the preceding claims, characterized by using as the said positive selecting antibody, an antibody which is directed against growth factor receptors on the membrane of normal cells, for example the EGF-receptor, PDGF (A and B) receptor, insuline receptors, insuline-like receptors transferrin receptor, NGF and FGF receptors.

5. Method according to one of the preceding claims, characterized by using an antibody directed against the group of integrins and other adhesion membrane molecules, and MDR proteins in normal cells.

6. Method according to one of the preceding claims, characterized by directing the antibody or fragments thereof against antigen or receptors in cells with abnormal developmental patterns, preferably such as primary and metastatic cancer cells.

7. Method according to one of the preceding claims, characterized by using as the said target-cell associating antibodies, antibodies of the IgG isotype, or $F(ab')_2$ or F(ab) fragments, or IgM, or fragments of IgM.

8. Method according to one of the preceding claims, characterized by preparing the mentioned cell suspension from mixed cell populations comprising mammalian tissues, for examples human bone marrow and peripheral blood, from pleural and peritoneal effusions, other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from solid tumors in normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissue, central nervous system, prostatic gland, skin and mucous membranes.

9. Method according to one of the preceding claims, characterized by that the antibody or antibody fragments is directed against groups of antigen determinants, such as those listed in the Table 1 of the specification.

10. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody or antibody fragment which is directed against growth factor receptors and oncogene products expressed on the membrane of malignant cells, for example insuline receptors, insuline-like receptors and FGF receptors in addition to those listed in Table 1 of the specification.

11. Method according to one of the preceding claims, characterized by using an antibody or antibody fragment directed against the group of integrins, other adhesion membrane molecules and MDR proteins in abnormal cells as listed in Table 1.

12. Method according to one of the preceding claims, characterized in that the used antibodies, antibody fragments

or combinations of these are directed to the antigen determinants as listed in Table 1 of the specification.

13. Method according to one of the preceding claims, characterized by using as the said antibody an antibody which is reactive with antigens present on abnormal cells, for example breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma and cancer cells of the gastrointestinal and genitourinary tract, and of the reticuloendothelial system, and/or target-cells associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune gastrointestinal, genitourinary, reticuloendothelial and other disorders.

14. Use of the detection method according to one of the preceding claims, for isolation of target-cells, whereby the complex of cells and the paramagnetic particles are exposed to a magnetic field and the resulting magnetically aggregated cells are further subjected to biological, biochemical and immunological examinations, including also characterisation of specific genes at the DNA, mRNA and protein level, including polymerase chain reaction (PCR) and reverse transcriptase PCR.

15. Use of the method for detection of specific target-cells according to one of the preceding claims, whereby it is established in vitro cell cultures from the separated paramagnetic particle-target-cell-complexes, and/or for inoculation into immunodeficient animals, preferably to establish human tumor xenografts in the said animals.

16. Kit for performing the method according to one of the preceding claims, characterized by that it comprises;
1, specific antibodies or antibody fragments directed to the antigen receptors on the wanted target-cells, where said antibody or antibody fragment is bound or can be bound to included paramagnetic particles, without removing their antigen-binding ability, and/or

2, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc portions of the target-cell associating antibodies, and specific free target-cell antibodies, and/or

3, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc-portions of the target-cell associating antibodies, bound to specific anti-target-cell antibodies, and/or

4, other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target cells, where said antibodies or antibody fragments are conjugated to biotin, peroxidase, alkaline phosphatase, or other enzymes, or where said antibodies or antibody fragments are bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

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(54) Title: IMPROVED METHOD FOR DETECTION OF SPECIFIC TARGET CELLS IN SPECIALIZED OR MIXED CELL POPULATION AND SOLUTIONS CONTAINING MIXED CELL POPULATIONS

(57) Abstract

The invention relates to a method for detecting specific target-cells in a simple and time saving way, using paramagnetic particles, antibodies recognizing the Fc portions of target-cell associating antibodies and target-cell associating antibodies directed to specific antigen determinants in the target-cell membranes. Incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method. The method can further be used for isolation of the target-cells by magnetic field application and a kit for performing the method according to the invention is described.

Improved method for detection of specific target cells in specialized or mixed cell population and solutions containing mixed cell populations

The present invention relates to an immunomagnetic method for detection of specific target cells in cell populations and solutions of cell populations. The invention also relates to a kit for performing the method in different cell populations.

In biology, biochemistry and adjacent fields it is considerable need for methods in which chemical entities are linked together. Such methods have an increasing importance in research regarding both normal and abnormal cell populations. Especially when solid supports are used cells can be immobilized, detected and isolated and purified. Furthermore, the cell content may be examined using a range of sofisticated methods. It is also of importance to be able to isolate the cells in viable forms.

Affinity binding is a sofisticated way of linking chemical/biochemical entities together. In such a method a pair of binding partners, which for example are attached to the substances to be linked, bind to each other when brought in contact. One of the binding partners in such a linkage may be represented by a molecule on the cell surface. Several such binding partner systems are known, such as antigen- antibody, enzyme- receptor, ligand- receptor interactions on cells and biotin- avidin binding, of which antigen-antibody binding is most frequently used. A hapten/anti-hapten binding pair method has also recently been suggested (PCT/EP90/01171).

When such methods are used for isolation of specific cells, which are the subject for further various examinations, it is necessary to reverse the linkage without producing destructive effects on the binding partners, which ideally should recover their function upon returning to the original conditions. This is not always the case, although it is proposed a method for

adequately cleaving antigen/anti-antigen and hapten/anti-hapten linkages (PCT/EP91/00671, PCT/EP90/01171).

Methods are known in which one of the binding partners is attached to an insoluble support, such as paramagnetic particles, and by which isolation of target cells in a mixed cell population is performed as negative isolation or positive isolation. In a negative isolation procedure the unwanted cells can be removed from the cell preparation by incubating the cells with antibody-coated particles, specific for the unwanted cells. Following the incubation the cell/antibody/particle-complex can be removed using the particles, leaving the wanted target cells behind. This result is often not satisfactory, since the wanted cells are left in the cell population, more or less purified, and also since the intention of the isolation procedure is to examine and/or perform further studies on the specific target cells. Attempts have been made to use particles for positive isolation, in which the wanted target cells are removed from the mixed cell population. These procedures have, however, been directed to certain target cells and are not suited for all target cell systems. A positive isolation procedure involving the hapten/anti-hapten linkage system has recently been proposed (PCT/EP90/01171) and also a method for isolating haemopoietic progenitor cells from bone marrow (PCT/EP90/02327). The latter is directed to use a combination of positive and negative selection for the purpose of isolating and possibly growing specific cells, i.e. haematopoietic progenitor cells, in the bone marrow, and is dependent upon removal of the particles.

PCT/EP90/01171 relates to a method of connecting target cells to an insoluble support by using the abilities of hapten, anti-hapten antibodies and anti-cell antibodies to bind to each other, thus constructing a linkage between the insoluble support, i.e. particle, and the target cell, consisting at least of hapten and anti-hapten antibody or combinations of hapten and anti-hapten antibodies and anti-anti-hapten antibodies or secondary anti-cell antibodies. The later

cleavage of the complex is performed by again exposing it to hapten or hapten analogue. Thus the constructed link always consists of hapten in addition to 1 or more elements. The method is directed to unspecified target cells and is directed to isolation of target cells and release of the insoluble support.

There is a need for a simple linkage to connect a target cell to an insoluble support, which do not involve compounds of a rather unspecified haptene-group, and which is directed to detection of specific target cells, with a minimum of unspecific cell association and which render unnecessary a later cleavage between the insoluble support and the specific target cell.

Thus the object of the present invention is to detect for diagnostic purposes specific target cells when used in a blood and bone marrow, without the problem with unspecific binding to normal cells. It represents a sensitive detection method for a variety of cell types, such that a high number of cells can be readily screened in the microscope and the procedure is rapid and simple. Furthermore, the present method can be used for isolation of cells for biochemical, biological and immunological examination, and for studying of specific genes at the nucleotide or protein level, in addition to culturing the cells, without the need for cleaving the cell-particles complex. A further object of the invention is to provide a kit for performing the method as characterized in the claims.

The intensions of the inventions are obtained by the method and kit characterized in the enclosed claims.

The method for immunomagnetic detection of target cells in a mixed cell population and physiological solutions containing cell populations is suitable for detection, but may also be used in positive isolation of specific types of both normal cells and patogenic cells. The method creates a linkage between a specific target cell and an insoluble support, such as

paramagnetic particles , which consists of one or two elements. The particle is either coated with an anti-cell antibody of murine or human origin, directed to the specific antigen determinants in the membranes of the wanted target-cells, or the particles are coated with a polyclonal anti-mouse or anti-human antibody capable of binding to the Fc-portions of the specific anti-cell antibody directed to the antigen determinants in the target-cell membranes. Instead of using the polyclonal anti-mouse/anti-human antibody for coating the particles, a monoclonal rat anti-mouse/anti-human antibody may be used. This last antibody, due partly to its monoclonal origin, may provide a more specific binding to the anti-cell antibody and reduce the risk for possible cross-reactions with other cells in solutions, such as blood. Furthermore, incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method.

In the following a more detailed disclosure of the method is presented, using cancer cells as the target-cells for detection and possible isolation. The method is, however, not limited to cancer cells and the disclosure shall not limit the method to this particular field of use, since the method is suitable within a range of cytological research areas.

In the management of cancer patients, the staging of the disease with regards to whether it is localized or if metastatic spread has occurred to other tissues, is of utmost importance for the choice of therapeutic alternative for the individual patient. Malignant cells spread by direct invasion into the surrounding tissue, through the lymphatics or by the distribution of tumor cells in the blood to distant organs, including the bone marrow and the central nervous system and the cerebrospinal fluid.

Detection of metastatic tumor cells has, until recently, relied on morphological methods using light and electron microscopy on biopsied tumor specimens, on smears of bone marrow and peripheral blood, and on slides prepared after cytosentrifugation of various body fluids. Since the advent of monoclonal antibodies recognising antigens predominantly expressed on the surface of different types of malignant cells, the identification of metastatic cells has, to an increasing extent, also involved immunocytochemistry and immunofluorescence. Thus, slides prepared from biopsied tumors or cytosentrifugates have been treated with monoclonal antibodies, and the binding of these to the tumor cells is visualized colorimetrically or by fluorescence. The latter method requires the use of a fluorescence microscope, alternatively preparing a cellsuspension an use a flow cytometer.

The previous methods suffer from limited sensitivity and/or specificity, and is usually laborious and time consuming, also requiring a high degree of expertise. Flowcytometric examiniations also involve expensive equipment.

The morphological methods for the detection of tumor cells in blood and bone marrow are much less sensitive than methods involving immunocytochemistry and immunofluorescence (Beiske et al., Am. J. Pathology 141 (3), September 1992). Also the latter methods are, however, inadequate in cases where the tumor cells represent less than 1 % of the total number of nucleated cells. Flow cytometry may provide better sensitivity than the methods involving the use of a microscope, but requires the availability of a high number of cells, and also involves several technical difficulties. Thus, aggregation of cells may cause problems, and the method does not provide possibilities to distinguish between labeled tumor cells and unspecifically fluorescing normal cells.

The invention allows for a very sensitive detection of, for example, metastatic tumor cells, since a high number of cells

can readily be screened in the microscope and the attached magnetic beads are easily recognisable. The monoclonal antibodies used bind with sufficient specificity to, for example, tumor cells and not to other cells than the target-cells present in mixed cell suspensions, like blood, bone marrow, and in other tumor manifestations, such that all cells with attached beads represent the target-cells. In addition, the procedure is rapid and simple, and can be performed by any investigator with access to a conventional microscope.

The novel method involves the binding of monoclonal antibodies, e.g. of murine or human origin, that specifically recognize antigens present on tumor cells, and not on the normal cells in question, or for other purposes to specified subpopulations of normal cells, to paramagnetic particles, either directly or to beads first covered with antibodies specifically recognizing the respective antibodies, or the Fc-portion of IgG antibodies, that bind to the tumor cells. The cell binding antibodies may be of the IgG or IgM type or being a fragment of ab IgG or IgM. Examples of used anti-target-cell antibodies may be those directed against groups of antigen determinants, for example CD56/NCAM antigen (MOC-1), Cluster 2 epithelial antigen (MOC-31), Cluster 2 (MW~40kD) antigen (NrLu10) (Myklebust et al. Br. J. Cancer Suppl. 63, 49-53, 1991), HMW-melanoma-associated antigen (9.2, 27) (Morgan et al., Hybridoma, 1, 27-36, 1981), 80kD, Sarcoma-associated antigen (TP1 & TP3) (Cancer Res. 48, 5302-5309, 1988), mucin antigens (Diel et al., Breast Cancer Res. Treatm., 1991), or EGF-receptor antigen (425.3) (Merck), in addition to the anti-pan-human antibody (Bruland et al., unpublished), which is suitable for detecting human cells among animal cells. The 425.3 antibody is directed towards antigens in both normal and malignant cells. Antibodies can furthermore be directed against growth factor receptors, for example EGF-receptor, PDGF (A and B) receptor, insuline receptor, insuline-like receptor, transferrin receptor, NGF and FGF receptors, group of integrins, other adhesion membrane molecules and MDR proteins in both normal cells and abnormal cells, and antigens present on subpopulations of normal cells,

in addition to oncogenic products, expressed on the membranes of normal and malignant cells and on malignant cells alone, for example Neu/erb B2/HER2. As for the malignant cells, these may be breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma, cancer cells of the gastrointestinal tract and the reticuloendothelial system, or the target-cells may be associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune, gastrointestial, genitourinary, reticuloendothelial and other disorders. Furthermore, the malignant cell population may be located in bone marrow, peripheral blood, come from pleural and peritoneal effusions and other body fluid compartments, such as urine, cerebrospinal fluid, semen, lymph or from solid tumors in normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissue, the central nervous system, prostatic gland, skin and mucous membranes. A more complete list of the antigen determinants and the corresponding antibodies or antibody fragments used in the present improved method is presented in Table 1.

The method comprises attachment of the antibodies directly to the paramagnetic particles, or the attachment can take place by attachment to surface-bound antibodies, such as polyclonal anti-mouse antibodies, monoclonal rat anti-mouse antibodies or monoclonal anti-human antibodies, specifically recognizing the Fc-portion of the said individual antibodies. The antibody-coated paramagnetic beads are then mixed with the suspension of cells to be examined and incubated for 5-10 min to 2 h, preferably for 30 min at 0-25°C, preferably at 4°C, under gentle rotation. The present method may also be performed in a changed order of steps, in that the free target-cell antibodies are added to the cell suspension, incubated for 5-10 min to 2h, preferably 30 min, at 0-20°C, preferably 4°C, under gentle rotation. The paramagnetic particles, precoated with anti-mouse or anti-human antibodies are then added to the incubated cell suspension, as described above, and the resulting suspension subjected to a further incubation of 5-10 min to 2h, preferably 30 min, at 0-25°C, preferably 4°C under gentle agitation.

Samples of the cell suspension are then transferred to a cell counting device, and the fraction of cells with attached beads relative to the total number of cells is determined under light microscopy. The number of antibody-coated beads added to the cell suspension should be between 0.5-10 times the number of target cells. When this number is unknown, the amount of coated beads added should be 1-10 % of the total number of cells.

For specific purposes, and in the cases where the density of the target-cells is low, for example malignant cells, or the target-cells represent a very low fraction of the total number of cells (≤ 1%), the target cells can be positively separated from non-target cells in a magnetic field. The isolated target cells, can then be enumerated microscopically and the fraction of target cells relative to the total number of cells in the initial cell suspension can be calculated. Moreover, the target-cells may be characterized for the presence of specific biochemical and biological features. Of particular importance will be the use of such cells for studies in molecular biology. In contrast to the above cited methods of the prior art, the present method allows studies and growth of the target-cells without performing a cleavage of the paramagnetic particle-target cell linkage. For several purposes it is of interest to examine specific genes in a pure population of target cells at the DNA, mRNA and protein level, both in tumor biopsies as well as in tumor cells present in blood, bone marrow and other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from otherwise normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissues, central nervous system, prostatic gland, skin and mucous membranes, and in other areas of cytological research activity.

With the methods of prior art, signals obtained on Southern, Northern and Western blots represent the normal cells as well as the tumor cells in the biopsy. If a single cell suspension is first prepared from the tumor material, and the tumor cells are then positively immunomagnetically detected and separated, any gene studies performed on this material would represent the

target-cells only. This also relates to for example malignant cells present in mammalian tissues, for example in bone marrow, peripheral blood, pleural and peritoneal effusions, and other body fluids, for example urine, cerebrospinal fluid, semen and lymph. Studies involving polymerase chain reaction (PCR) methodology will also gain in specificity and reliability when performed on pure tumor cell populations obtained by the new method.

The application of the new method steps may differ depending on type of tissues to be examined.

a) Tissue from solid or needle tumor biopsies is prepared mechanically or with mild enzymatic treatment into a single cell suspension, to which the primary, specific antibodies or antibody fragments are added directly or after washing the cell suspension with phosphate buffered saline or culture medium with or without serum, such as fetal calf serum, bovine, horse, pig, goat or human serum.

b) If the material is a sample of pleural or ascitic effusion, cerebrospinal fluid, urine, lymph or body fluids such as effusions in the joints of patients with various forms of arthritis, the specific antibodies or antibody fragments are either added to the samples directly, or after centrifugation with or without washings before or after the cells in the samples are spun down and brought back into suspension.

c) If the material consists of blood or bone marrow aspirate, the mononuclear cell fraction is isolated by gradient sentrifugation on e.g. Lymphoprep before washing, resuspension, and addition of the appropriate antibodies or antibody fragments.

The procedure conditions for a) and b) are established, as exemplified by results obtained in successful experiments as those described below.

For c) the results have been found to be influenced by a high number of factors which have been examined in detail. Among these are antibody concentration, the ratio of the number of paramagnetic particles versus number of cells, incubation times and volumes, type of incubation medium, and the pH level. The particle to mononuclear cell ratio in all experiments should be in the range of 0.5/1 - 2/1, depending on the binding affinity of the primary specific antibodies or fragments.

A major problem has been unspecific attachment to normal blood or bone marrow cells of particles coated with either sheep or rat anti-mouse antibodies alone, or in addition with the specific antibodies. Experiments have shown that the unspecific binding is equally high without the presence of the specific antibodies, indicating that the problem is not caused by cross-reactivity of the targeting antibodies to normal cells. The possibility that the less than optimal specificity could be caused by ionic binding has been ruled out. Another possibility was that subpopulations of normal cells of the B-lineage might adhere to the particle-antibody complexes. However, immunomagnetic removal of B-cells from the cell suspension before adding the specific antibodies/antibody-particle complexes did not improve the specificity of the latter.

The problem with the procedure used on isolated mononuclear fractions of bone marrow and peripheral bllod, that some non-target cells might also bind paramagnetic particles, has been circumvented or overcome. Thus with sheep-anti-mouse antibody coated particles alone or with specific antibodies the number of particles unspecifically attached to a low fraction mononuclear blood or bone marrow cells was reduced from an average of 10 to about 1 and in paralell the fraction of normal cells with particles decreased from 1-2% to 0.5-1% or less.

Evidence has been obtained that the problem may be caused by hydrophobic forces associated with the antibodies bound to the paramagnetic particles. Methods for reducing this hydrophobicity is thus claimed. One such method is

preincubation of the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, for example Tween 20 in concentrations of less than 0.1% for 30 minutes at 4°C. When possible selection of the target cells is warrented, the cell suspension should contain a low concentration of the detergent, e.g. 0.01% of Tween 20. In several experiments this procedure has almost eliminated or dramatically reduced the problem of unspecific binding seen with the mononuclear cell fractions from blood or bone marrow.

The other improvement which, if found warrantied, may be used together with the detergent step as follows:

After incubation of the cell suspension with the primary antibodies or antibody fragments and the antibody-coated paramagnetic particles as described in previously, the cell suspension is incubated with a second set of antibodies or antibody fragments directed against other extracellular or against intracellular determinants of the target cells, with our without pretreatment with cell fixatives such as formaldehyde or alcohols. These antibodies or their fragments should have been prelabeled by fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or enzymes like peroxidase and alkaline phosphatase, allowing visualization by per se known methods in the microscope and/or a suitable counting device.

The target cells will both be visualized with the latter method and have bound particles to their surface, and can thus be enumerated.

To simplify the distinction between non-target and target cells, the cell suspension can before the second visualization step either be subjected to cytospin centrifugation or portions of the suspension are attached to caoted glass slides on which the particle-bound cells will be spread out in a thin layer, facilitating the recognition of the double-"stained" cells.

For use in the new procedure, kits will contain for example precoated paramagnetic particles prepared for each monoclonal antibody. In another embodiment the kits contain paramagnetic particles pre-coated with IgG isotype specific anti-mouse or anti-human antibody as one part of it, and different target cell-associated, for example tumor cell, antibodies as another part. In a third embodiment the kit contains paramagnetic particles precoated with specific anti-Fc antibodies, such as polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-mouse, or anti-human antibodies, capable of binding to the Fc-portion the target-cell associating antibodies, bound to specific anti-target-cell antibodies. In a further embodiment the kit contains other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target-cells, where said antibodies or antibody fragments are conjugated to peroxidase, alkaline phosphatase, or other enzymes, together with relevant substrates to such enzymes, or where said antibody or antibody fragment is bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

The present method will in the following be illustrated by model experiments, examples of the usefulness of the new method and examples of practical applications. These examples shall not be regarded as in any way limiting the invention.

Model experiments:

1. Binding of antibody-bead complexes to tumor cell lines with the new procedure:

To determine antibody concentrations and optimal conditions for the binding of antibody-paramagnetic particle complexes to tumor cells, a large panel of cancer cell lines was used. The paramagnetic beads were bound to the cells, either by coating the specific antibodies to sheep-anti-mouse antibody (SAM)-coated paramagnetic particles, or by first incubating the cells with the specific antibodies, washing, followed by a second

incubation with SAM-coated particles. The results of these experiments are given in Tables 2a and 2b, in which + indicates binding of several beads to all cells, (+) indicates either a lower number of beads bound to each cell, or that not all the tumor cells had beads attached to their surface, whereas - reflects no binding, and (-) indicates very weak binding.

2. For detection of tumor cells in the mononuclear fraction of bone marrow or peripheral blood, model experiments were performed where specific antibodies and SAM-coated paramagnetic particles were added either to such mononuclear cells or to a cell suspension where a different number of cancer cells from in vitro cultivated cell lines were added to said mononuclear cells. In some experiments, either the mononuclear cells, or the malignant cells were prestained with a fluorescent dye, to be able to distinguish beteween the two types of cells. In all experiments, non-binding primary antibodies, and/or sheep-anti-mouse antibody-coated beads were used separately as controls.

Table 2a

Antibodies		Cell lines							
		MCF-7	SKBR3	T47D	MDA231	MDA435	DU145	FMEX-1	LOX
NrLu10	IgG2b		+	+	(+)	(+)	+		
Moc31	IgG1	+	+	+	(+)	(+)	+		
Moc1	IgG1			(+)	(+)	+			
12H12	IgG1		+	+		+	+		
2E11	IgG3	+	+	+		+	+		
5A6	IgG1		(+)	+					
5F2	IgM			(+)					
CC3	IgG2a	-	-	-				-	
CC1	IgM			-				(+)	
CU18	IgG1	-	-	-					
CU46	IgG1	(+)	-	-					
7F11	IgG1	-	-	+			-	-	- -
D7	IgG3			(+)					
E4SF	IgG1?		+	+			(-)	-	50%+
425-3				+				-	+
9.2.27								+	+
MUC18			-				-	-	-
2g12	IgG1							+	
4b7	IgG1							+	
BM2 (=2F11)									
BM7 (=7F11)									
TP-3									
TP-1									
CEA									
GINTES	IgG								
3C9	IgM								
HH8	IgM								
5F4	IgM								
3F1	IgG1								

Table 2b

Antibodies		Cell lines							
		PM1	MA-11	CRL1435	CRL1740	H-146	Colo205	786-0	WIDR
NrLu10	IgG2b	+	+	+	+	+	+	-	
Moc31	IgG1	+	+	+	+	+	+	+	+
Moc1	IgG1					+	-		
12H12	IgG1	+	+	(+)		-	-	-	
2E11	IgG3	(+)	+	-	+	-	-	-	
5A6	IgG1	+	+						
5F2	IgM								
CC3	IgG2a					-		-	
CC1	IgM					(+)		-	
CU18	IgG1					-		-	
CU46	IgG1					-		-	
7F11	IgG1	(+)	+	-		-	-		-
ID7	IgG3					-			-
E4SF	IgG1?	+	+	+	+	-	-		-
425-3									
9.2.27									
MUC18		-							
2g12	IgG1					-		-	
4b7	IgG1					-		-	
BM2 (=2F11)		+	+						
BM7 (=7F11)		+							
TP-3									
TP-1									
CEA									
GINTES	IgG					+			-
3C9	IgM					-			-
HH8	IgM					-			-
5F4	IgM					-			-
3F1	IgG1					-			-

In several experiments some unspecific binding to the mononuclear cells was observed, which was found to be unrelated to the nature of the specific antibody, and which was equally pronounced with SAM-coated particles alone. The magnitude of this unspecific binding varied from almost 0 to a level between 0.5-2%. This unspecific binding was almost eliminated by mild treatment with detergent, (Tween 20) performed to reduce the problem of hydrophobic cell interactions.

EXAMPLES OF THE USEFULNESS OF THE NEW PROCEDURE

1. Detection of micrometastic neoplastic disease in blood and marrow

Early and reliable diagnosis of spread of cancer cells to blood and/or bone marrow has become increasingly important for the choice of optimal therapy, possibly curative in many types of cancer, including carcinomas, as described in application Example 1. Similar procedures for malignant melanoma, sarcoma, neuroblastoma and several other cancers have been established or are under development.

2. Detection of malignant cells in pleural or ascitic effusions, and in urine

The nature of such effusions may represent an important diagnostic problem, particularly when a low number of cancer cells are present together with normal reactive or epithelial cells. In several cases a definite diagnosis has been rapidly made with the new method, in cases where conventional cytological examination has been negative or inconclusive. A similar advantage can be found in cases of cancer in the kidneys or in the urinary tract and bladder.

3. Detection of neoplastic cells in the cerebrospinal fluid

As the systemic treatment of many cancer types have improved, the frequency of cases with symptom-giving brain metastases have significantly increased, and in parallell with this, the necessity for early detection of such spread. With the use of the new procedure even a low number of malignant cells can easily be identified, permitting intervention with therapeutic alternatives at an early stage of intracranial tumor manifestations.

4. Diagnosis of cancer in biopsied tissue

When cancer is suspected, and tissue biopsies are obtained by surgical procedures or by e.g. needle biopsies, a much more simple and rapid diagnosis can be made with the new method, used on prepared cell suspensions, compared to conventional morphological or immunohisto- or cytochemical procedures.

Distinction between several alternative cancers can be made by the use of the appropriate antibodies.

5. Identification of prognostic indicators

Since the expression of several membrane molecules have been shown to correlate with progression of the malignant disease in several cancers, the present method can be used to identify prognostic indicators, for example as described in application Example 2.

6. Identification of cells indicative of specific diseases or of disease progression or state

In various types of rheumatoid diseases (such as rheumatoid arthritis), as well as in allergic, autoimmune, and cardiovascular diseases, identification of the systemic or local presence of specific subpopulations of cells is important

for diagnosis and for determining the stage of the disease. Rapid detection of such cell populations with the new method is therefore of considerable diagnostic and therapeutic importance.

7. Detection of subpopulations of normal cells

For several purposes, it will be important to detect the fraction of a particular subpopulation of normal cells in a population. This applies e.g. to liver biopsies where the identification of cells expressing the biliar epithelial antigen, may be of importance. Similarly, the identification, and possible isolation of specific endothelial cells from a cell suspension prepared from various normal tissues may be warranted.

Several of the cell membrane molecules mentioned in sections 1-6 may also be used as targets for immunotherapy with several types of activiated killer cells or e.g. with immunotoxins. The identification with the new method of expression of such molecules is, therefore, also of value for determining in which cases such types of therapy should be used.

Examples of a practical application of the method:

Example 1

To diagnose spread of cancer cells in blood and/or bone marrow at an early stage, we have used in the new procedure the MOC-31, NrLu10, BM2, BM7, 12H12, and MLuC1 anti-carcinoma antibodies to determine whether or not micrometastatic disease from breast, lung, colorectal, and prostate cancer might be sensitively identified in such body fluids. The successful results with these antibodies have significant clinical implications.

Example 2

The expression of serveral cell membrane molecules have been shown to correlate with progression of the malignant disease in

several types of cancer. The detection of binding of such antibodies to respective antibodies can therefore be used to obtain information of high prognostic value. Among such antigens are a high number of adhesion molecules, carbohydrate antigens, glycolipids, growth factor receptors and carcinoma markers listed below. We have, with the new procedure identified the binding of particle-antibody complexes to CD44-variants, E-cadherin, Le^y, CEA, EGF-r, transferrin receptor, MUC-1 epitope, LUBCRU-G7 epitope, prostate cancer antigen, UJ13A epitope, β_2-microglobulin, HLA-antigens, and apoptosis receptor.

Example 3

Two litres of pleural diffusion from a patient supposed to suffer from malignant melanoma was obtained. After centrifugation, the cells were suspended in a volume of 2 ml of RPMI with a 10% fetal calf serum, incubated with 9.2.27 anti-melanoma antibody (10 µg/ml) at 4°C for 30 min, washed and again incubated with Dynabeads SAM M450/IgG2A at 4°C for 30 min. The cell suspension was then examined under a microscope for determining the fraction of cells with paramagnetic cells attached to their surface. The diagnosis of malignant melanoma was confirmed, as about 10% of the cells had a significant number of particles rosettes.

Example 4

Biopsied tissue was obtained from a subcutaneous tumor in a case with clinical indications of either small cell lung cancer or a malignant melanoma. A single cell suspension was prepared from the biopsy, divided in 2 fractions, one incubated with the 9.2.27 anti-melanoma antibody, and the other with MOC-31 anti-carcinoma antibody (both at 10 µg/ml). The incubation was similar to that used in the example above. None of the cells incubated with the melanoma antibody bound any beads, whereas all tumor cells incubated with MOC-31 were positive.

Example 5

Biopsied tissue from a patient suspected to have malignant melanoma was examined by preparing single cell suspension, incubating with 9.2.27 anti-melanoma antibody, and then following the procedure as above. Most of the cells were positive with a high number of particle-rosettes attached to their membranes.

Example 6

A pleural effusion from a breast cancer patient was studied to examine whether tumor cells could be detected in the fluid. One litre of the fluid was centrifuged, the cells resuspended, and in separate vials incubate with each of 3 different anti-carcinoma antibodies (MOC-31, 2E11, 12H12). After completing the procedure as in the previous example, it was found that most of the cells bound to antibody-coated particles in all 3 cases.

Example 7

A bone marrow suspension obtained from a breast cancer patient was studied to examine whether micrometastic tumor cells could be present. After the preparation of mononuclear cells, these were incubated with the same 3 anti-carcinoma antibodies used in the example above, but in this case the antibodies were first attached to Dynabeads SAM IgG paramagneteic particles. After 1 incubation with these directly coated particles, the cell suspension was examined in the microscope, and a high number of cells were found positive with a number of particle-rosettes attached to their membrane.

Similar experiments have been performed in a number of pleural or ascitic effusion and bone marrow from patients with breast cancer.

Example 8

T47D human breast carcinoma cells were incubated for varying lenghts of time with Hoechst fluoresence dye, and the viability of the labeled cells was checked. Varying numbers of labeled breast carcinoma cells were then added to 1 x 10^6 bone marrow

cells obtained from healthy volunteers. In different experiments, different concentrations of paramagnetic, monodisperse particles (Dynabeads P450) coated with individual anticarcinoma antibodies (NrLu10, MOC31, or 12H12) were added. After incubation for 30 min on ice, samples of the different test tubes were examined in a counting chamber under light and fluorescence microscopy. When the ratio of tumor cells/total nucleated cells was low, the cell suspension was subjected to a magnetic field and the cells with particles attached were isolated before examined in the microscope. It was found that at an optimal ratio of 1-10 paramagnetic beads per tumor cell in the cell mixture, all the tumor cells had from 2-15 beads attached to their surface. The sensitivity of the detection method was close to one target-cell per 10^4 nucleated cells. In control experiments with labeled tumor cells using antibodies known to have some cross-reativity to normal cells, this cross-reactivity was confirmed with the antibody-coated paramagnetic particles. In experiments with beads without tumor-associated antibody coating, none of the target cells bound any beads.

Similar experiments have been performed both with other breast cancer lines and a small cell lung cancer cell line. Similar sensitivity and specificity were obtained in these experiments.

Example 9

Pleural and ascites fluid from patients with breast cancer and ovarian carcinoma were sentrifuged, the same coated paramagnetic particles used in Example 1 were added, incubated and concentrated in a magnetic field before the suspension was examined under light microscopy. Typically, cells that had the clear morphological features of tumor cells had beads attached, whereas none of the few normal cells bound the antibody-coated beads. In two cases with pleural effusion, an independent morphological examination did not reveal the presence of any tumor cells, whereas a significant number malignant cells were detected by the use of anibody-coated beads. In some cases, tumor cells were separated in a magnetic field and transferred

to tissue culture flasks containing growth medium specially prepared for growing breast cancer cells, in attempts to establish permanent cell lines from these cultures. In parallel, cells from the malignant effusions were cultivated directly without positive selection with magnetic beads. In the latter cases, no cell line could be established, whereas in more than 50 % of the cases where positively selected tumor cells had been used, cell lines were successfully established.

Example 10

In some cases, bone marrow and peripheral blood obtained from patients with breast cancer were examined with the present procedure by adding antibody-coated paramagnetic beads, incubating for 30 min at 4°C and concentrating in a magnetic field and by examining the suspension under light microscopy. In both cases binding of the paramagnetic beads to tumor cells, representing 0,1-1 % of the nucleated cells in the bone marrow and blood was detected, cells that could not be identified by any other method.

Example 11

Antibodies against certain growth factor receptors or other gene products expressed on the surface of specific cell populations may be used to identify and positively select these cells. Beads coated with anti-transferrin receptor antibodies, used in the novel method according to the present invention were shown to represent a rapid, simple and sensitive method for identification of cells expressing the transferrin-receptor.

Example 12

For various purposes isolation of specific populations of normal cells is warranted. Endothelial cells lining the capillary or small vessels in normal or tumorous tissue could be positively selected from cell suspensions prepared from the relevant tissues. The procedure involved the use of beads coated with antibody directed against structures expressed on

the endothelial cells, but not on the other normal cells in the cell mixture.

Example 13

Human cells injected into immunodeficient rodents was shown to be present in cell suspensions prepared from tumor xenografts and from various host organs/tissues by employing magnetic particles coated with an anti-pan human antibody.

Table 1

LIST OF RELEVANT ANTIGENS AND EXAMPLES OF ASSOCIATED ANTIGEN-BINDING ANTIBODIES

ANTIGENS	MONOCLONAL ANTIBODIES
Adhesion molecules	
Fibronectin receptor ($\alpha5\beta1$ integrin)	Pierce 36114, BTC 21/22 Calbiochem 341649
Integrin $\alpha3\beta1$	M-Kiol 2
Vitronectin receptor ($\alpha v\beta3$ integrin)	TP36.1, BTC 41/42
Integrin $\alpha2$	Calbiochem 407277
Integrin $\alpha3$	Calbiochem 407278
Integrin $\alpha4$	Calbiochem 407279
Integrin $\alpha5$	Calbiochem 407280
Integrin αV	Calbiochem 407281
Integrin $\beta2$	Calbiochem 407283
Integrin $\beta4$	Calbiochem 407284
$GpII\beta III\alpha$	8221
ICAM-I (CD54)	C57-60, CL203.4, RR 1/1[1]
VCAM-1	Genzyme 2137-01
ELAM-1	Genzyme 2138-01
E-selectin	BBA 8
P-selectin/GMP-140	BTC 71/72
LFA-3 (CD58)	TS 2/9
CD44	BM 1441 272, 25.32
CD44-variants	11.24, 11.31, 11.10
N-CAM(CD56)	MOC-1
H-CAM	BCA9
L-CAM	BM 1441 892
N-CAM	TURA-27
MACAM-1	NKI-M9
E-cadherin	BTC 111, HECD-1, 6F9
P-cadherin	NCC-CAD-299
Tenascin	BM 1452 193, Calbiochem 580664
Thrombospondin receptor (CD36)	BM 1441 264
VLA-2	A1.43
Laminin receptor	
HNK-1 epitope	HNK-1
Carbohydrate antigens	
T-antigen	HH8, HT-8
Tn-antigen	TKH6, BaGs2
Sialyl Tn	TKH-2

Table 1 (cont.)

Gastrointestinal cancer associated antigen (M$_r$200kD)	CA 19-9
Carcinoma associated antigen	C-50
Le^y	MLuC1, BR96, BR64
di-Le^x, tri-Le^x	B3
Dimeric Le^x epitope	NCC-ST-421
H-type 2	B1
CA15-3 epitope	CA15-3
CEA	I-9, I-14, I-27, II-10, I-46, Calbiochem 250729
Galb1-4GlcNac (nL4,6,8)	1B2
H-II	BE2
A type 3	HH8
Lacto-N-fucopentanose III (CD15)	PM-81
Glycolipids	
GD_3	ME 36.1, R24
GD_2	ME36.1, 3F8, 14.18
Gb_3	38-13
GM_3	M2590
GM_2	MKI-8, MKI-16,
$FucGM_1$	1D7, F12
Growth factor receptors	
EGF receptor	425.3, 2.E9, 225
c-erbB-2 (HER2)	BM 1378 988, 800 E6
PDGFα receptor	Genzyme 1264-00
PDGFβ receptor	Sigma P 7679
Transferrin receptor	OKT 9, D65.30
NGF receptor	BM 1198 637
IL-2 receptor (CD25)	BM 1295 802, BM 1361 937
c-kit	BM 428 616, 14 A3, ID9.3D6
TNF-receptor	GEnzyme 1995-01, PAL-M1
NGF receptor	
Melanoma antigens	
High molecular weight antigen (HMW 250.000)	9.2.27, NrML5, 225.28, 763.74, TP41.2, IND1
Mw105 melanoma-associated glycoprotein	ME20
100 kDa antigen (melanoma/carcinoma)	376.96
gp 113	MUC 18
p95-100	PAL-M2
Sp75	15.75
gr 100-107	NKI-bereb
MAA	K9.2
M$_r$125kD (gp125)	Mab 436
Sarcoma antigens	
TP-1 and TP-3 epitope	TP-1, TP-3

Table 1 (cont.)

M_r 200kD	29-13, 29.2
M_r 160kD	35-16, 30–40
Carcinoma markers	
MOC-31 epitope (cluster 2 epithelial antigen)	MOC-31, NrLu10
MUC-1 antigens (such as DF3-epitope (gp290kD))	MUC-1, DF3, BCP-7 to -10
MUC-2 and MUC-3	PMH1
LUBCRU-G7 epitope (gp 230kD)	LUBCRU-G7
Prostate specific antigen	BM 1276 972
Prostate cancer antigen	E4-SF
Protate high molecular antigen $M_r > 400$kD	PD41
Polymorphic epithelial mucins	BM-2, BM-7, 12-H-12
Prostate specific membran antigen (Cyt-356)	7E11-C5
Human milk fat globulin	Immunotech HMFG-1, 27.1
42kD breast carcinoma epitope	B/9189
$M_r > 10^6$ mucin	TAG-72, CC-49, CC-83
Ovarian carcinoma OC125 epitope (m_r 750 kD)	OC125
Pancreatic HMW glycoprotein	DU-PAN-2
Colon antigen Co17-1A (M_r 37000)	17-1A
G9-epitope (colon carcinoma)	G9
Human colonic sulfomucin	91.9H
M_r 300kD pancreas antigen	MUSE11
GA 733.2	GA733, KS1.4
TAG 72	B72.3, CC49, CC83
Undefined	Oat1, SM1
Pancreatic cancer-associated	MUSE 11
Pancarcinoma	CC49
Prostate adenocarcinoma-antigen	PD 41
M_r 150-130kD adenocarcinoma of the lung	AF-10
gp160 lung cancer antigen (Cancer Res. *48*, 2768, 1988)	anti gp160
M_r 92kD bladder carcinoma antigen	3G2-C6
M_r 600kD bladder carcinoma antigen	C3
Bladder carcinoma antigen (Cancer Res. *49*, 6720, 1989)	AN43, BB369
CAR-3 epitop $M_r > 400$kD	AR-3
MAM-6 epitope (C15.3)	115D8
High molecular ovarian cancer antigen	OVX1, OVX2
Mucin epitope Ia3	Ia3
Hepatocellular carcinoma antigen M_r 900kD	KM-2
Hepema1 epitope (gp43) Hepatocellular carc. ag	Hepema-1
O-linked mucin containing N-glycolylneuraminic acid	3E1.2
M_r 48kD colorectal carcinoma antigen	D612
M_r 71kD breast carcinoma antigen	BCA 227
16.88 epitope (colorectal carcinoma antigen	16.88
CAK1 (ovarian cancers)	K1
Colon specific antigen p	Mu-1, Mu-2
Lung carcinoma antigen M_r 350–420kD	DF-L1, DF-L2

Table 1 (cont.)

gp54 bladder carcinoma antigen	T16
gp85 bladder carcinoma antigen	T43
gp25 bladder carcinoma antigen	T138
Neuroblastoma antigens	
Neuroblastoma-associated, such as UJ13A epitope	UJ13A
Glioma antigens	
Mel-14 epitope	Mel-14
Head and neck cancer antigens	
M_r18-22kD antigen	E48
HLA-antigens	
HLA Class 1	TP25.99
HLA-A	VF19LL67
HLA-B	H2-149.1
HLA-A2	KS1
HLA-ABC	W6.32
HLA-DR, DQ, DP	Q 5/13, B 8.11.2
β_2-microglobulin	NAMB-1
Apoptosis receptor	
Apo-1 epitope	Apo 1
Various	
Plasminogen activator antigens & receptors	Rabbit polyclonal
p-glycoprotein	C219, MRK16, JSB-1, 265/F4
cathepsin D	CIS-Diagnostici, Italy
biliary epithelial antigen	HEA 125
neurogiandular antigen (CD63)	ME491, NKI-C3, LS62
CD9	TAPA-1, R2, SM23
pan-human cell antigen	pan-H

CLAIMS

1. Improved method for detecting specific target cells in cell suspensions of mixed cell populations and in fluid systems containing mixed cell populations, and in single cell suspensions prepared from solid tissues, characterized by comprising the following steps:

1.1. coating, by a per ce known procedure, paramagnetic particles or beads with either, a) antibodies, or antibody fragments directed against membrane structures specifically expressed on target-cells and not on non-target-cells in the cell mixture or;
b) antibodies, preferably polyclonal anti-mouse or monoclonal rat anti-mouse antibodies or anti-human antibodies, capable of binding to the Fc-portions of the said antibodies, directed against the membrane structures; and

1.2.1. mixing the target-cell-associating antibodies (murine or human) which is attached to the said particles or beads, or attached to the beads pre-coated with anti-mouse or antihuman antibodies recognizing the Fc-portions of the target-associating antibodies, with the cell suspension containing the target-cells, or,

1.2.2. mixing free target-cell-associating antibodies with the cell suspension containing the target cells and incubate this mixture for 5-10 min to 2 h, preferably 30 min, at a temperature between 0°C and 20°C, preferably 4°C under gentle rotation, and;

1.3. incubating the mixture of the cell suspension and target-associating antibodies attached to paramagnetic particles or beads (1.2.1), or paramagnetic particles or beads, precoated with anti-mouse or anti-human antibodies recognizing the Fc-portion of the target-associating antibodies, to the mixture of incubated free target associating antibody and cell suspension (1.2.2.), and incubating, for 5-10 min to 2 h,

preferably 30 min, at a temperature between 0°C and 25°C, preferably 4°C, under gentle rotation, and;

1.4.1. if the target cell population is contained in blood or bone marrow aspirates the hydrophobic forces associated with antibody-coated particles are reduced by pre-incubating the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, e.g. Tween 20 in concentrations less than 0.1% for 30 min at 4°C, and/or

1.4.2. by incubating the cell suspensions, untreated or pretreated with formalin, alcohol or other fixatives, with other antibodies or antibody fragments binding to extracellular or intracellular molecules present in the target cells and the antibodies used are labeled in advance by peroxidase, alkaline phosphatase, or other enzymes permitting visualization of the binding by addition and incubation with relevant substrates, or

1.4.3. the antibody fragments are biotinylated and the binding visualized when adding the incubating with avidin complexed to peroxidase, alkaline phosphatase, or other enzymes, with addition and incubation with relevant substrates, or

1.5.1. subjecting the incubated paramagnetic particle-antibodies-cell mixture (1.3) to a magnetic field if the density of target-cells is low, or if the ratio of target cell/total cells in the cell mixture is low (≤ 1 %) and then examining and counting the stained or unstained particle-target-cell complexes in the cell suspension, using a micropscope and/or a suitable cell/particle counting device, or,

1.5.2. examining and counting the target-cells in the incubated mixture of paramagnetic particles, antibodies and cell mixture (1.3), or in the case when the antibodies or antibody fragments are conjugated to non-paramagnetic particles that can be visualized directly because of colour or through enzymatic activation, using a microscope and/or a suitable cell/particle

counting device if the ratio of target-cells/total cells in the cell suspension is adequate (> 1 %).

2. Method according to claim 1, characterized by directing the antibody or fragments thereof against the antigens in normal, living cells, such as liver hepatocytes, Kupffer cells and endothelial cells type 1 and 2 and Clara cells of the lung, endothelial cells of specific organs, pancreatic exocrine and endocrine cells, kidney tubule cells, bladder epithelial cells, brain glial and ependymal cells, bladder and prostate epithelial cells, ciliated cells of airways, different subpopulations of mucosal cells in the gastrointestinal tract, pituitary cells, and other endocrine cells in various hormone-producing organs.

3. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody which is reactive with antigens present on subpopulations of normal cells and oncogenic products expressed on the membrane of normal tissue cells.

4. Method according to one of the preceding claims, characterized by using as the said positive selecting antibody, an antibody which is directed against growth factor receptors on the membrane of normal cells, for example the EGF-receptor, PDGF (A and B) receptor, insuline receptors, insuline-like receptors transferrin receptor, NGF and FGF receptors.

5. Method according to one of the preceding claims, characterized by using an antibody directed against the group of integrins and other adhesion membrane molecules, and MDR proteins in normal cells.

6. Method according to one of the preceding claims, characterized by directing the antibody or fragments thereof against antigen or receptors in cells with abnormal developmental patterns, preferably such as primary and metastatic cancer cells.

7. Method according to one of the preceding claims, characterized by using as the said target-cell associating antibodies, antibodies of the IgG isotype, or $F(ab')_2$ or F(ab) fragments, or IgM, or fragments of IgM.

8. Method according to one of the preceding claims, characterized by preparing the mentioned cell suspension from mixed cell populations comprising mammalian tissues, for examples human bone marrow and peripheral blood, from pleural and peritoneal effusions, other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from solid tumors in normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissue, central nervous system, prostatic gland, skin and mucous membranes.

9. Method according to one of the preceding claims, characterized by that the antibody or antibody fragments is directed against groups of antigen determinants, such as those listed in the Table 1 of the specification.

10. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody or antibody fragment which is directed against growth factor receptors and oncogene products expressed on the membrane of malignant cells, for example insuline receptors, insuline-like receptors and FGF receptors in addition to those listed in Table 1 of the specification.

11. Method according to one of the preceding claims, characterized by using an antibody or antibody fragment directed against the group of integrins, other adhesion membrane molecules and MDR proteins in abnormal cells as listed in Table 1.

12. Method according to one of the preceding claims, characterized in that the used antibodies, antibody fragments

or combinations of these are directed to the antigen determinants as listed in Table 1 of the specification.

13. Method according to one of the preceding claims, characterized by using as the said antibody an antibody which is reactive with antigens present on abnormal cells, for example breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma and cancer cells of the gastrointestinal and genitourinary tract, and of the reticuloendothelial system, and/or target-cells associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune gastrointestinal, genitourinary, reticuloendothelial and other disorders.

14. Use of the detection method according to one of the preceding claims, for isolation of target-cells, whereby the complex of cells and the paramagnetic particles are exposed to a magnetic field and the resulting magnetically aggregated cells are further subjected to biological, biochemical and immunological examinations, including also characterisation of specific genes at the DNA, mRNA and protein level, including polymerase chain reaction (PCR) and reverse transcriptase PCR.

15. Use of the method for detection of specific target-cells according to one of the preceding claims, whereby it is established in vitro cell cultures from the separated paramagnetic particle-target-cell-complexes, and/or for inoculation into immunodeficient animals, preferably to establish human tumor xenografts in the said animals.

16. Kit for performing the method according to one of the preceding claims, characterized by that it comprises;
1, specific antibodies or antibody fragments directed to the antigen receptors on the wanted target-cells, where said antibody or antibody fragment is bound or can be bound to included paramagnetic particles, without removing their antigen-binding ability, and/or

2, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc portions of the target-cell associating antibodies, and specific free target-cell antibodies, and/or

3, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc-portions of the target-cell associating antibodies, bound to specific anti-target-cell antibodies, and/or

4, other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target cells, where said antibodies or antibody fragments are conjugated to biotin, peroxidase, alkaline phosphatase, or other enzymes, or where said antibodies or antibody fragments are bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/[redacted]93/00136

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: G01N 33/53, C12Q 1/00, C12N 5/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOTECHNOLOGY

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9204961 (IMMUNICON CORPORATION), 2 April 1992 (02.04.92), see examples 2-4 and in particular col. 14, lines 62-68	16
Y		1-16
	--	
Y	WO, A1, 9109938 (HOLMES MICHAEL JOHNET AL), 11 July 1991 (11.07.91), see the examples	1-16
X		16
	--	
X	WO, A1, 9101368 (HOLMES MICHAEL JOHN ET AL), 7 February 1991 (07.02.91)	16
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Information on patent family members

27/11/93

International application No.
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WO-A1- 9109938	11/07/91	AU-A- 7069791	24/07/91
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EP-A2- 0403960	27/12/90	AU-A- 5717990	20/12/90
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